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THE DIFFERENTIAL DETERMINATION OF CATECHOLAMINES IN URINE[1]

B. D. DRUJAN, T. L. SOURKES, D. S. LAYNE,[2] AND G. F. MURPHY

Abstract

Details of uniform procedures for the routine determination of adrenaline, noradrenaline, and dopamine in urine are given. The first two compounds are determined by a trihydroxyindole method; for dopamine, a modification of the method of Carlsson and Waldeck is used. Determination of adrenaline and noradrenaline is illustrated using three different types of fluorometer. Normal values for the 24-hour excretion of these compounds in the urine of normal adults are in micrograms: adrenaline, 16.4 $\mu g \pm 1.1$, noradrenaline, 54.9 $\mu g \pm 3.2$, dopamine, 198.8 $\mu g \pm 36.2$ (mean $\pm$ standard error). Some examples of the levels of catecholamine excretion in various clinical states are given.

Introduction

The application of the principles of fluorometry to the determination of the catecholamines (CAs) has proved most valuable in the study of the biochemistry and physiology of the sympathoadrenal system. An extensive methodological literature has grown up around this subject, and the prospective researcher in this field has numerous techniques from which to choose. These techniques, in fact, resolve themselves into two distinct methods, distinguished by the reactions employed to prepare fluorescent derivatives from the CAs (1): the trihydroxyindole method (2, 3) stemming from the work of Ehrlen (4) and Lund (5); and the ethylene diamine (ED) condensation method (6, 7), based upon the reaction discovered by Natelson and his colleagues (8). Both methods are widely used now, the high sensitivity of the ED method lending itself especially to the measurement of CAs in blood. In this laboratory a routine procedure for the determination of the CAs in urine and tissues was worked out some time ago (1) employing the principles of the trihydroxyindole method, and has been in use for over two years. In that time it has been modified in two directions: on the one hand, the volume of materials handled for the estimation has been reduced appreciably; on the other hand, the procedure has been expanded to permit the differential estimation of adrenaline and noradrenaline. Three different styles of fluorometric instrument have been tested, and the

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similarity of the results obtained further indicates the utility of the procedure in routine work. The determination of dopamine (3-hydroxytyramine) according to Carlsson and Waldeck (9) is also feasible under similar conditions to those used for adrenaline and noradrenaline, and the principles described by the investigative group in Lund have been incorporated in the present procedure. Because the fluorescent derivative of dopamine absorbs light in the ultraviolet frequencies and, in fact, fluoresces near 400 mμ, this CA can be estimated only with an instrument containing a quartz (or equivalent) optical system. The purpose of this paper is to describe the simplified technique for the extraction and determination of CA found in urine.

Methods

1. Extraction of Catecholamines

Urine is collected in acid (5 ml glacial acetic acid or hydrochloric acid diluted with 5 ml of water for a 24-hour specimen) and is stored in the refrigerator. To a 10-ml aliquot of the pooled urine, measured into a 50-ml test tube, is added: 2.5 ml of 10% ethylenediaminetetraacetic acid, 0.5 g of neutral alumina of chromatographic grade (e.g. Woelm "non-alkaline" alumina[3]), and 1 drop of 1% phenolphthalein. The mixture is brought to approximately pH 8 (light pink color) by the careful, successive addition of 5 *N*, 2.5 *N*, and 1.25 *N* sodium hydroxide, with constant swirling of the suspension. After twice shaking (mechanical shaker) for 5 minutes (with adjustment of pH in between) the alumina is allowed to settle and the supernatant is removed by means of suction (water pump) applied through a fine-bore pipette. A suspension of the moist alumina in 2.5 ml of 0.2 *M* sodium acetate is shaken for 10 minutes and the supernatant is discarded as before. This is repeated with glass-distilled water. The adsorbed CAs are eluted with 3.5 ml of 0.5 *M* acetic acid (15-minute shaking). The acidic eluate is decanted, centrifuged, and preserved for the CA estimation.

2. Formation of Fluorescent Derivatives

A. From Adrenaline and Noradrenaline

Two aliquots, 0.4 and 0.8 ml, of the eluate are added to 15×150 mm test tubes. The volume is made up to 1.0 ml with glass-distilled water. Two milliliters of pH 6 buffer (1 *M* sodium acetate adjusted to pH 6 with 1 *M* acetic acid) is added. After they are mixed, the contents of the tubes are oxidized with 0.5 ml of 0.009 *N* iodine solution for exactly 3 minutes. The reaction is then stopped by the addition of 0.5 ml of 0.01 *N* sodium thiosulphate. Lutin formation is brought about by the addition of 1.0 ml of 4.5 *N* sodium hydroxide containing 2 mg ascorbic acid. The samples are allowed to stand for 15 minutes before readings are taken. Their fluorescence is then found to be proportional to the amount of CA present in the tube. Readings made earlier than this give erratic results, probably because of the existence of other fluorescent species which eventually decompose, leaving only the lutins. Calibration factors based upon 0.05–0.3 μg of CA are determined when the eluates are carried through

[3]Alupharm Chemicals, 322–28 Lafayette Street, New Orleans, Louisiana.

the fluorometric procedure. Fluorescence may be determined in either a filter or a grating instrument. As already pointed out (1), dopamine contributes some fluorescence (about 4% as much as an equivalent amount of noradrenaline) which is detected with the filter combination employed in the Farrand fluorometer. Dopamine interference is even less when the spectrophotofluorometer is used. However, if the concentration of dopamine is high, as occurs in certain urines, the contribution of this CA to the total fluorescence using pH 6 buffer may be substantial.

B. Differential Estimation of Adrenaline

Procedure 2A serves in the measurement of total CAs, primarily adrenaline and noradrenaline, as a single entity. Adrenaline can be differentiated from its congeners by conducting procedure 2A in duplicate, using Sørensen's glycine–HCl buffer, 0.1 *N*, pH 3, for the second set. At pH 3 and under the oxidative conditions described neither noradrenaline nor dopamine contributes appreciably to the fluorescence ultimately determined, so that, in effect, adrenaline is determined alone. Fluorescence may be estimated as in procedure 2A with either a filter or grating fluorometer.

C. Determination of Dopamine

A convenient aliquot of the eluate from alumina is made up to 1.0 ml with 0.5 *N* acetic acid. The blank tube receives 1.0 ml of 0.5 *N* acetic acid. Two milliliters of pH 6 acetate buffer is added. Oxidation is carried out by adding 0.5 ml of 0.009 *N* iodine solution, terminating its action after 3 minutes by the addition of 0.5 ml of 4.5 *N* NaOH containing 12.6 mg anhydrous sodium sulphite. After a further 3 minutes, 1.0 ml of 5 *N* HCl is added. The samples are allowed to stand for 45 minutes. During this time interfering fluorescent species decay and stable readings are obtained which are proportional to the concentration of dopamine. Fluorescence is determined using a grating fluorometer. The lutins of adrenaline and noradrenaline are also formed during this oxidation but do not interfere at the wavelengths at which dopamine is measured.

3. Recovery of Catecholamines

The losses in CAs occurring at various stages in the adsorption and elution from alumina must be taken into account. The recovery of adrenaline (or other CA) is evaluated by adding 0.5 μg, 1.0 μg, and 1.5 μg of the compound to three 10-ml samples of urine. These tubes are then assayed along with the set to be determined. The values found for them are corrected for the amount of adrenaline estimated to be originally present, by subtracting the value for the corresponding urine samples.

4. Measurement of Fluorescence

(a) Farrand Model A fluorometer.—The primary filter is made up of two Corning glasses, No. 5113 (blue) and No. 3389 (pale yellow). This combination selects the 436 mμ line of the mercury vapor lamp source (10). The secondary filter is a yellow Corning glass No. 3486. The galvanometer is adjusted to

100 mm deflection using a solution of quinine sulphate (or of some other stable fluorescent substance).

(b) Coleman electronic photofluorometer.—The primary filter is Coleman No. 12-224; secondary filter is Coleman No. 14-212. The scale is adjusted in the same way as for the Farrand instrument.

(c) Aminco-Bowman spectrophotofluorometer.—Adrenaline and noradrenaline differ somewhat in the wavelengths at which they fluoresce maximally; some investigators have taken advantage of this property to set up the necessary two sets of conditions for measuring these compounds differentially in this way. When the concentration of CAs is very low, as in plasma, this system may prove useful for maintaining the amines as concentrated as possible in the eluates. In this laboratory the preference has been to activate adrenaline- and noradrenaline-containing solutions at 420 mμ and to read the fluorescence intensity at 510 mμ. A yellow Corning filter (No. 3385) is inserted between the secondary grating and the phototube to reduce the amount of scattered light from the cuvette compartment. The widest slit arrangement for this compartment is used. The wavelength settings for the estimation of dopamine (procedure 2C) are 330 mμ and 375 mμ, respectively. All wavelengths cited are uncorrected (11). The data given here were obtained by using the RCA 1P28 photomultiplier tube.

5. Calculations

The calculations for adrenaline (procedure 2B) and dopamine (procedure 2C) are straightforward, inasmuch as each of these two compounds is measured without interference from the other or from noradrenaline. The estimation of noradrenaline is made on the basis of its conjoint contribution to fluorescence at pH 6 (procedure 2A), corrected for the adrenaline and, if necessary, the dopamine present. The amount of adrenaline present in an "unknown" (μg in the aliquot of eluate taken for estimation) is

$$A = \frac{100\ M_3}{P_a \times F_{3a}}$$

where M_3 = observed reading for sample at pH 3,
P_a = percentage recovery of added adrenaline,
F_{3a} = calibration factor for adrenaline at pH 3 (scale deflection per μg adrenaline).

When the value for "A" has been calculated, the amount of noradrenaline in the "unknown" is

$$N = \frac{100\ M_6}{P_n \times F_{6n}} - \frac{(F_{6a} \times P_a)\ A}{P_n \times F_{6n}}$$

where M_6 = observed reading at pH 6,
P_n = percentage recovery of added noradrenaline, at pH 6,
F_{6n} = calibration factor for noradrenaline at pH 6,
F_{6a} = calibration factor for adrenaline at pH 6.

Inasmuch as all the symbols above are constants for a given run, except M_3, M_6, A, and N, the equations may be simplified to the following:

$$A = k_1 M_3, \qquad \text{where } k_1 = \frac{100}{P_a \times F_{3a}};$$

$$\text{and } N = k_2 M_6 - k_3 A, \qquad \text{where } k_2 = \frac{100}{P_n \times F_{6n}}$$

$$\text{and } k_3 = \frac{F_{6a}}{F_{6n}} \times \frac{P_a}{P_n}.$$

The noradrenaline value can be corrected for dopamine, using its calibration factor, F_d, in the same way as shown for adrenaline.

Results

Calibration Factors

The list of calibration factors in Table I indicates the range obtained on the three instruments used at various times in this work. The ratios, $F_{3a} : F_{6a} : F_{6n}$,

TABLE I

Catecholamine calibration factors*

Instrument	No. detns.	F_{3a}	F_{6a}	F_{6n}	F_d
Farrand Model A fluorometer	20	756±28† (100)‡	227±17 (30.0)	233±9 (30.7)	
Aminco-Bowman spectrophotofluorometer	26	21.8±0.4 (100)	4.2±0.1 (19.3)	11.0±0.4 (50.6)	5.2-6.6§
Coleman electronic photofluorometer	2	635 (100)	114 (18)	280 (44)	

*Deflection of galvanometer needle in arbitrary units per microgram of CA.
†Mean ± standard error.
‡Ratio of factors, the value F_{3a} being taken as 100.
§Four determinations.

show that similar optical analyses are obtained with the spectrophotofluorometer, which selects a narrow band of wavelengths from the spectrum of the xenon arc lamp and from the emission spectrum of the irradiated sample, and the Coleman photofluorometer, which makes use of colored filters with relatively wide bands. This contrasts with the results using the Farrand Model A fluorometer. Whereas the other two instruments allow the estimation of noradrenaline with about 2.5 times the sensitivity of adrenaline (at pH 6), this fluorometer measures the two compounds with equal sensitivity. It is possible, of course, that the use of a different secondary filter in the Farrand fluorometer would alter these ratios in favor of noradrenaline.

Recovery of Catecholamines

The data in Table II show that the percentage recovery is the same for adrenaline and noradrenaline, and is similar to values found by other investigators.

TABLE II

Percentage recovery of catecholamines added to urine

	No. detns.	Adrenaline* Mean	Adrenaline* S.E.	Noradrenaline† Mean	Noradrenaline† S.E.
Present procedure‡	26	70.5	1.9	69.1	3.0
von Euler and Orwen (12)	10	85	2	—	—
	27	—	—	78	2
Pitkänen (13)	20	76.1	—	—	—
	62	—	—	69.5	—
Kärki (14)	6	72.0	2.0	72.7	1.7
Griffiths and Collinson (15)	19	—	—	92	2
Weil-Malherbe and Bone (16)§	11	82.6	2.8	87.9	2.2

*Estimated at pH 3 (procedure 2B).
†Estimated at pH 6 (procedure 2A).
‡The Aminco-Bowman spectrophotofluorometer was used in obtaining the above data. Recovery of dopamine (18 determinations) was 60%.
§Recovery of added dopamine was 90.7 ± 4.0%.

Normal Urinary Values for Catecholamines

These are shown in Table III. The mean values for men and women are very similar; the over-all means (± standard error) and ranges for 27 subjects (45 determinations) are: adrenaline, 16.4 μg (±1.1 μg), 3.2–31.0 μg; and noradrenaline, 54.9 μg (±3.2 μg), 25.4–130.4 μg. In nine women and four men

TABLE III

Twenty-four-hour excretion of catecholamines in the urine

Clinical state	Sex	No. subjects	No. detns.*	Mean ± S.E. (μg/24 hr) Adrenaline	Noradrenaline
Normal adults	F	15	28	15.4±1.7	52.7±3.3
	M	12	17	17.9±2.0	58.7±6.7
Pheochromocytoma					
Patient (U.B.)	F				
Before removal of tumor:					
33 days before				249	3204
4 days before				46	209
2 days before				142	618
After operation:					
8 days after				9	15
110 days after				4	2
Malignant melanoma					
Patient G.	M		2	31.2	144.8
Patient D.	M		1	14.6	92.7
Patient M.	M		1	24.2	114.4
Hyperthyroidism					
Patient (E.K.)	F		2	12.8	34.2
Kidney disease					
Patient M.J.†	F		1	8.9	14.9
Patient F.M.‡	M		1	8.8	16.0
Hepatolenticular degeneration					
Patient V.M.	F	1	6	12.1	24.6
Familial dysautonomia	M	1	2	18.0	49.5
Neurotic depressive reaction	F	9	20	16.7±2.2	62.9±5.4
	M	1			
Anxiety reaction	F	3	14	16.0±4.6	56.2±3.6
	M	4			

*Each determination represents the analysis of a separate 24-hour urine.
†Chronic pyelonephritis.
‡Membranous glomerulonephritis (non-nephrotic stage).

(18 determinations) the mean 24-hour output of dopamine was 198.8 μg, (±36.2 μg), and the range was 26–595 μg. Noradrenaline excretion exceeded 100 μg/24 hr on only two occasions in our normal series. For comparison with values given by certain authors in terms of mμg per ml of urine, the normal levels in the present series were (mean and standard error): adrenaline, 14.0 mμg ±3.3; noradrenaline, 47.5 mμg ±12.1; dopamine, 183.7 mμg ±33.9.

Catecholamine Excretion in Disease States

The urinary levels of CAs in a number of clinical conditions is also shown in Table III. The high values in the urine of the patient with pheochromocytoma contrast markedly with the low levels found after the excision of a large chromaffin tumor from the right suprarenal gland. The values for the noradrenaline fraction in three cases of malignant melanoma were high. Because of the disordered catechol metabolism in this disease it is necessary to determine if this fluorescence is due solely to noradrenaline or to some other compound as well. Noradrenaline values were low in two cases of kidney disorder, as well as in one of hepatolenticular degeneration (being treated with penicillamine at the time the analyses were performed). In two groups of psychiatric patients urinary adrenaline and noradrenaline levels were normal.

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PROTECTION AGAINST ACUTE TRAUMA AND TRAUMATIC SHOCK BY VASODILATORS[1]

MARK NICKERSON AND STEFAN A. CARTER[2]

Abstract

The effects of hydralazine and Dibenzyline, agents which induce vasodilatation by different mechanisms, on the development of shock were studied in rats subjected to two levels of trauma in a Noble–Collip drum. Hydralazine effectively reduced the incidence of acute traumatic deaths (those occurring during or within 15 minutes after completion of the period of trauma), but it protected against late, shock deaths and improved over-all survival only among animals subjected to relatively mild trauma. Dibenzyline completely eliminated all late, shock deaths and increased over-all survival at both levels of trauma. The total incidence of early deaths was unaltered, but among Dibenzyline-treated animals a higher percentage of these occurred during the period of trauma, indicating some increased susceptibility to the immediate effects of acute trauma. The drug also caused some increase in the incidence of intraperitoneal hemorrhage due to rupture of the liver or spleen. Vascular engorgement of these organs, which increased during the drumming period in both control and Dibenzyline-treated animals, was not altered by the drug. The results of combined administration of hydralazine or polyvinylpyrrolidone (PVP) and Dibenzyline were qualitatively and quantitatively the same as those obtained with Dibenzyline alone. These data indicate that vasodilatation induced by inhibition of sympathetic vasoconstriction provides effective protection against death from shock, but may increase susceptibility to the immediate deleterious effects of acute trauma. A drug such as hydralazine, which can increase cardiac output, and acute expansion of plasma volume by PVP may sustain the circulation, particularly the cerebral circulation, during acute trauma, but do not effectively increase ultimate survival.

One of the most prominent responses to various types of shock-inducing stress is the development of intense vasoconstriction in various vascular beds. This may be considered to be a compensatory mechanism designed to prevent collapse by supporting the blood pressure and the cerebral and coronary circulations, and it probably helps to sustain the performance of the organism under conditions of acute stress. However, several lines of evidence suggest that this reflex vasoconstriction may contribute significantly to the development of shock (see (1)), and severe, prolonged vasoconstriction may itself induce typical "irreversible" shock (2, 3, 4). The areas of maximal vasoconstrictor tone during the development of shock have not been clearly defined, nor has the area(s) in which vasoconstriction is most deleterious been established with certainty, although the splanchnic bed has been strongly implicated (5).

Inhibition of vasoconstriction by sympathectomy (6), deafferentation of traumatized areas (7), or adrenergic or ganglionic blocking agents (8, 9, 10) has been shown to provide varying degrees of protection against the development of irreversibility. These agents and procedures all induce vasodilatation by reducing sympathetic vasoconstrictor tone, and consequently, the anatomical

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[2]Postdoctoral Medical Research Fellow of the National Research Council of Canada.

distribution of their effects is determined by the sympathetic tone during the development of shock. No data are presently available to indicate whether simple inhibition of sympathetic vasoconstrictor tone produces a distribution of vasodilatation which is optimal for the prevention of death from shock.

The present experiments were undertaken to compare the antishock properties of an effective and relatively specific adrenergic blocking agent (phenoxybenzamine, Dibenzyline) and a vasodilator drug with very different mechanisms of action (hydralazine, Apresoline). The latter probably produces vasodilatation primarily by a direct action on the vascular smooth muscle, but an action on the central nervous system also may be involved (11, 12). Hydralazine increases cardiac output and visceral blood flow in normovolemic animals and in man, but has only a slight effect on blood flow to the extremities (12, 13, 14). It has very little adrenergic blocking activity and does not block sympathetic ganglia.

Methods

All experiments were performed on female albino rats of a "Wistar" strain, which were fasted about 18 hours prior to drumming, but had free access to water. Body weights varied between 125 and 155 g, but the range within any one group averaged only 12 g and animals of different weights were randomized between the experimental and control groups. In most experiments, the animals were subjected to 400 or 450 turns in a Noble–Collip drum with two shelves (15). However, because of observed variations in the number of falls per revolution, 900 turns in a drum with one shelf was substituted in some of the later experiments. Controls were subjected to trauma concurrently with the various experimental groups and comparisons were made only between control and experimental animals subjected to trauma on the same days. Animals from the various groups were alternated between two essentially identical drums mounted on the same shaft. All control and experimental groups were composed of three to five subgroups run on different days over a period of several weeks to minimize the effects of possible unknown variables. The animals were observed closely for several hours after drumming and then returned to their cages. Those alive 48 hours after the end of the period of trauma were considered to be survivors. Gross post-mortem examinations were performed on all fatalities, and those deaths associated with intraperitoneal hemorrhage due to rupture of the liver or spleen were classified separately. Drugs were administered intraperitoneally in a volume of approximately 0.5 ml, usually 1 hour prior to drumming. Control animals received a comparable volume of 0.9% NaCl solution at the same time. In one series of experiments 3.0 ml of polyvinylpyrrolidone (PVP) was injected via a tail vein 15 minutes prior to drumming.

In experiments designed to determine the basis for apparent alterations in the incidence of rupture of the liver or spleen in some groups of treated animals, pretreated and control rats were subjected to the standard tumbling procedure and, after varying periods of trauma, were removed from the drum and quickly

immersed in liquid nitrogen. They were dissected prior to thawing, and the weights of the liver and spleen recorded as percentage of body weight.

Results

The effects of hydralazine and Dibenzyline on the mortality rate resulting from the standard tumbling procedure have been analyzed as effects on (*a*) over-all mortality, (*b*) incidence of acute traumatic deaths (early deaths, occurring during or within 15 minutes after the end of the period of trauma), (*c*) incidence of death due to traumatic shock (late deaths), and (*d*) incidence of intraperitoneal hemorrhage due to rupture of the liver or spleen.

Effects of Drugs on Over-all Mortality

Premedication with 2.0 mg/kg of hydralazine, given intraperitoneally 1 hour before tumbling, reduced mortality due to 400 turns of the Noble–Collip drum from 76% to 24% ($P < 0.01$) (Table I). However, at a more severe level

TABLE I

Effects of drug treatment on response to drum trauma

		Over-all mortality		Hemorrhagic deaths excluded			Acute deaths excluded	
No. turns	Group	No. animals	Total deaths, %	No. animals	Over-all mortality, %	Acute traumatic deaths, %	No. animals	Shock deaths, %
400	Control	21	76	21	76	19	17	71
	Hydralazine (2.0 mg/kg)	21	24	21	24	0	21	24
	P		<0.01		<0.01	<0.2		<0.01
450	Control	20	90	18	89	50	9	77
	Hydralazine (1.0–5.0 mg/kg)	28	75	26	73	15	22	68
	P		>0.3		>0.3	<0.05 (<0.01)*		>0.5
400	Control	12	75	12	75	17	10	70
	Dibenzyline (1.5 mg/kg)	12	33	11	27	27	8	0
	P		<0.2		<0.1	>0.5		<0.02
450	Control	16	81	15	80	40	9	67
	Dibenzyline (1.5 mg/kg)	16	50	11	27	27	8	0
	P		<0.2 (<0.02)*		<0.05 (<0.01)*	>0.5 (>0.5)*		<0.02 (<0.001)*
900†	Control	16	69	13	62	31	9	44
	Hydralazine (3.0 mg/kg) and Dibenzyline (1.5 mg/kg)	15	47	12	33	33	8	0

*P values in parentheses indicate significance when groups at 400 and 450 turns are combined.
†Drum with one shelf.

of trauma (450 turns), hydralazine did not protect. Doses of 1.0 to 5.0 mg/kg were tested in different subgroups of animals. The results obtained with these various doses are combined in the table because no differences between them were observed. Dibenzyline (1.5 mg/kg) intraperitoneally 1 hour before tumbling decreased the mortality at 400 turns from 75% to 33% and that at 450 turns from 81% to 50%. In contrast to the effects of hydralazine, the results obtained with Dibenzyline at the two levels of trauma are homogenous.

The protection provided by Dibenzyline in the combined groups is clearly significant ($P < 0.02$).

Exclusion of deaths associated with intraperitoneal hemorrhage due to rupture of the liver or spleen does not alter the calculated protection attributable to hydralazine at either 400 or 450 turns. However, the protection provided by Dibenzyline is more obvious when hemorrhagic deaths are excluded, the residual mortality being reduced by the drug from 75% to 27% at 400 turns and from 80% to 27% at 450 turns ($P < 0.01$).

Effects of Drugs on Incidence of Death Due to Acute Trauma

The most striking differences in the effects of hydralazine and Dibenzyline are apparent when the incidences of death due to acute trauma (early deaths) and those due to traumatic shock (late deaths) are analyzed separately (Table I). Deaths associated with intraperitoneal hemorrhage have been excluded. Pretreatment with hydralazine markedly decreased the incidence of early deaths at both levels of trauma, reducing the mortality during this period from 19% to 0 and from 50% to 15% respectively in the groups subjected to 400 and 450 turns. Although the number of animals involved was too small to allow definite conclusions, it appeared that the larger doses of hydralazine were more effective in preventing early deaths among animals subjected to the more severe trauma. All of the acute traumatic deaths in the hydralazine-treated group at 450 turns were among animals receiving 2.0 mg/kg or less of the drug. The marked effect of hydralazine on the incidence of traumatic death and its failure to reduce over-all mortality in the animals subjected to 450 turns can be reconciled by reference to Fig. 1, which shows the cumulative percentage mortality at various time intervals after drumming. Survival time among control animals varied from 0 (animals dead before the end of the drumming period) to about 40 hours. However, most of those alive 15 minutes after the end of the period of trauma survived for at least 2 hours. This hiatus was not present in the hydralazine-treated groups where 43% of all deaths occurred between 15 minutes and 2 hours after tumbling. These data indicate that an important effect of the hydralazine was to delay death. This was the major action in animals subjected to 450 turns, but was associated with a significant over-all protection in the groups subjected to the lesser trauma of 400 turns.

Pretreatment with Dibenzyline did not alter the incidence of deaths due to acute trauma. However, a considerably higher percentage of the early deaths occurred before the end of the period of drumming in the groups pretreated with Dibenzyline (Fig. 1), which suggests that the drug may have somewhat sensitized the animals to the immediate effects of the trauma.

Effects of Drugs on Incidence of Death Due to Traumatic Shock

The production and prevention of traumatic shock can be assessed properly only after exclusion of deaths due to acute trauma and to hemorrhage. When other causes of death are excluded (Table I) it is apparent that hydralazine provides significant protection against death due to traumatic shock in animals subjected to 400 turns of the Noble–Collip drum, but not against shock induced

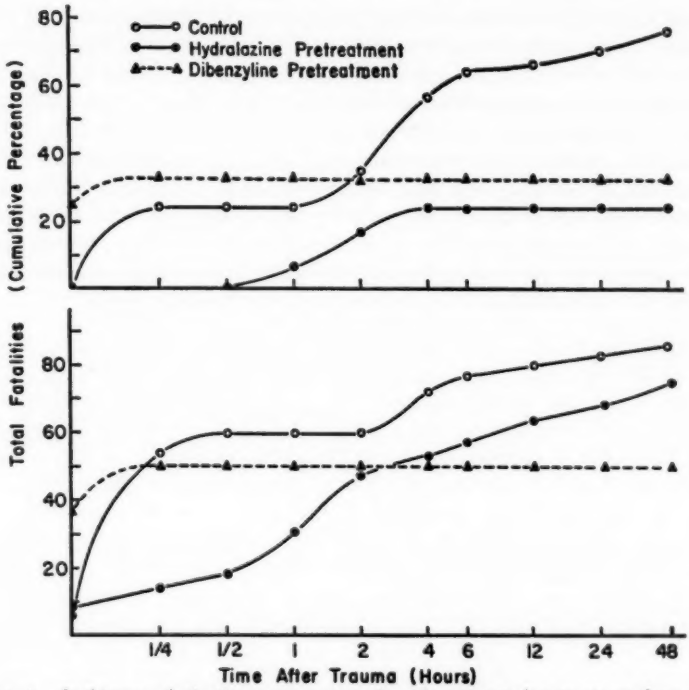

FIG. 1. Incidence of deaths among control and pretreated rats at various times following exposure to 400 (upper) and 450 (lower) turns in a Noble–Collip drum with two shelves. Plotted as cumulative percentage.

by the more severe trauma of 450 turns. These results must be assessed in relation to the observed protection against acute traumatic deaths. The prevention of early deaths shifts some of the more susceptible animals into the "traumatic shock death" group, which tends to obscure the protection against shock. In spite of this factor hydralazine significantly reduced the incidence of late deaths among animals subjected to 400 turns. In this group, no deaths occurred later than 4 hours after the period of trauma. The failure of hydralazine to alter over-all mortality among animals subjected to 450 turns indicates that the obvious delay in the time of death of many of the animals in this group did not mask any major protection against traumatic shock.

In contrast to hydralazine, Dibenzyline provided complete protection against death due to traumatic shock in the groups subjected to both 400 and 450 turns. All animals alive 15 minutes after the end of the period of trauma survived the procedure. In addition, these animals were alert and moderately active very shortly after completion of the drumming period, and showed little of the generalized depression and lethargy characteristic of even the ultimate survivors in the control and hydralazine-treated groups. The significant reduction in over-all mortality induced by Dibenzyline is clearly dependent on the protection against late shock deaths.

Effects of Combined Treatment with Hydralazine and Dibenzyline

Because hydralazine protected against acute trauma whereas Dibenzyline protected against traumatic shock, it appeared that combined administration of the two agents might provide greater over-all protection than either alone. However, administration of 3.0 mg/kg of hydralazine and 1.5 mg/kg of Dibenzyline 1 hour before drumming resulted in a higher mortality in the treated than in the control group. Administration of the hydralazine 1 hour and the Dibenzyline immediately before drumming also failed to provide augmented protection (Table I). However, it is interesting to note that all deaths among animals receiving the two drugs occurred early, indicating that the effect of the combination was dominated by the Dibenzyline.

Effects of Combined Treatment with Polyvinylpyrrolidone (PVP) and Dibenzyline

The probability that acute traumatic (early) deaths are due to an acute reduction in venous return, hypotension, and inadequate circulation to the respiratory centers suggested that acute expansion of the plasma volume might sustain the cerebral circulation during the period of trauma and thus complement the effect of Dibenzyline, which protects against late, shock deaths. All animals in this study were subjected to 900 turns in a drum with one shelf. Intravenous administration of 3.0 ml of PVP 15 minutes before tumbling, a volume equal to approximately 25% of the rats' estimated blood volume, appeared to provide some protection against early deaths, but did not alter over-all mortality (Table II). This effect of PVP was not apparent when the

TABLE II

Effects of dibenzyline and PVP on incidence of death due to acute trauma and to traumatic shock

Group	No. animals*	Traumatic deaths, %	Shock deaths, %	Survival, %
Control	14	64	14	22
PVP 3.0 ml/rat	7	29	57	14
Dibenzyline 1.5 mg/kg	4	25	0	75
PVP plus dibenzyline	8	50	0	50

*Animals with intraperitoneal hemorrhage from ruptured liver or spleen excluded.

plasma-volume expander was administered in combination with Dibenzyline (1.5 mg/kg intraperitoneally 1 hour prior to drumming). However, the PVP did not appear to interfere with the Dibenzyline protection against late deaths. Failure to obtain any suggestion of an additive or synergistic effect of PVP and Dibenzyline administered together in these experiments discouraged more extensive investigation of this combination.

Effects of Drugs on Incidence of Intraperitoneal Hemorrhage

Death was associated with rupture of the capsule of the liver or spleen and intraperitoneal hemorrhage in about 7% of all control animals. The incidence was somewhat lower among those subjected to 400 turns than among groups subjected to 450 or 900 (one shelf) turns, but the differences are not statistically significant, largely due to the relatively small numbers of animals with such

complications. In both the control and treated groups, rupture of the liver was a somewhat more frequent cause of intraperitoneal hemorrhage than damage to the spleen, accounting for about 60% of the total. Pretreatment with hydralazine did not alter the incidence of intraperitoneal hemorrhage. However, Dibenzyline caused some increase in all groups, and the over-all increase in incidence from 5% in the paired controls to 22% is clearly significant ($P < 0.02$).

Vasoconstriction and contraction of the spleen in response to sympathetic nervous system activity are components of the reaction to many types of stress, and it appeared possible that inhibition of these responses by Dibenzyline might cause engorgement of the liver and spleen and increase susceptibility to trauma. An attempt was made to assess the degree of vascular engorgement of the liver and spleen in control and Dibenzyline-treated animals at about the mid-point (425 turns) and near the end (850 turns) of a drumming period which would produce about 80% mortality. The animals were rapidly frozen in liquid nitrogen and the liver and spleen dissected in the frozen state.[3] The data obtained are presented in Table III, and show that the organ weights of

TABLE III

Effects of drum trauma and of dibenzyline on weights of liver and spleen

	No trauma			425 turns†			850 turns†		
Group	No. animals	Liver*	Spleen*	No. animals	Liver	Spleen	No. animals	Liver	Spleen
Control	5	3.82 ±0.24‡	0.44 ±0.04	6	4.86 ±0.29	0.58 ±0.07	4	4.64 ±0.22	0.45 ±0.07
Dibenzyline 1.5 mg/kg	5	3.69 ±0.18	0.34 ±0.03	6	4.83 ±0.27	0.61 ±0.03	6	4.43 ±0.28	0.43 ±0.02

*Organ weights expressed as percentage of body weight.
†Drums with one shelf.
‡Mean ± standard error.

the Dibenzyline-treated animals were not greater than those of the controls at any of the periods examined. Of even more interest is the fact that the weights of the liver and spleen did not decrease in either the control or treated animals during the period of trauma. Indeed, there was a consistent increase, most marked at the mid-point of the period of trauma. ($P < 0.05$ in the combined treated and control groups.) These data indicate the development of a considerable vascular engorgement of these organs during the drumming period. The fact that this change was not enhanced by pretreatment with Dibenzyline suggests that it was due to decreased sympathetic nervous system activity. This engorgement may predispose the liver and spleen to traumatic damage, but the data do not appear to provide an explanation for the increased incidence of damage in animals pretreated with Dibenzyline. The present studies do not rule out a possible local effect associated with the local irritant properties of β-haloalkylamines (16) as contributing to the increased incidence of intraperitoneal hemorrhage in animals treated with Dibenzyline by the intraperitoneal route.

[3]We are indebted to Mr. Harvey D. House for assistance with this phase of the investigation.

Post-mortem Findings

Except for occasional intraperitoneal hemorrhages associated with rupture of the capsule of the liver or spleen, the only gross pathological findings were associated with the gastrointestinal tract and lungs. The most prominent findings were vascular congestion of and/or hemorrhage into the wall and lumen of the intestine which were observed consistently in both control and hydralazine-treated animals, but were almost entirely absent in those treated with Dibenzyline. Evaluation of this differential is complicated by the fact that most deaths were earlier in the Dibenzyline-treated than in the other two groups. However, the absence of gastrointestinal pathology probably is attributable to the action of the drug because vascular congestion of the intestinal bed, although not hemorrhage, was a definite and consistent finding in the few control or hydralazine-treated animals which died during the period of trauma.

Hemorrhagic areas in the lungs were observed fairly frequently in all groups. The incidence did not appear to differ between control and treated animals, but this finding was somewhat more frequent in animals dying from the immediate effect of trauma (early deaths). No hemorrhages or other gross evidence of intracranial damage was found.

Discussion

Most of the many studies which have utilized the Noble–Collip drum to induce traumatic shock in rats, and occasionally in other species, have been evaluated in terms of over-all survival or death. However, separation of the fatalities into the groups employed above appears to be justified on the basis that the mechanisms of death probably are different. The causes of death appear to be most complicated in those animals in which intraperitoneal hemorrhage occurred. The hemorrhage probably acts as an additional stress superimposed on factors which led to death in 75 to 80% of the animals even without this complication. In the absence of any satisfactory method of quantitating the hemorrhage, or data on possible hemorrhages in survivors, death associated with intraperitoneal hemorrhage could only be tabulated as to total incidence.

Acute traumatic (early) deaths probably are due to factors very different from those involved in late, shock deaths. Such early deaths appear to result from rather sudden respiratory failure, probably secondary to acute hypotension and consequent medullary ischemia. Available reports are not in agreement regarding the blood pressure changes immediately following drum trauma. Chambers *et al.* (17), employing an indirect method of measurement, observed a marked, temporary hypotension immediately after drumming, although it appears that they excluded animals which died within 20 minutes after the completion of trauma. More recent direct measurements of blood pressure (18) failed to detect an early period of hypotension, although the recorded pulse contours indicated a reduction in venous return and in cardiac output. Unfortunately, no observations on animals dying shortly after completion of the period of trauma were included in this report.

It is tempting to attribute early deaths following drum trauma to mechanisms similar to those involved in the acute respiratory failure associated with periods of extreme hypotension during the production of hemorrhagic shock. In our experience, a short period of artificial respiration and return of the shed blood almost uniformly allows survival of animals in which respiratory arrest due to excessive hemorrhage has occurred early in the hypovolemic period. This easy reversibility clearly distinguishes early, acute hemorrhage from hemorrhagic shock, and although the picture is less clear-cut in animals subjected to trauma, a similar differentiation between early and late deaths appears possible.

In the present experiments, as has been reported previously (15), the more advanced pathological changes in the intestinal tract characteristic of shock (petechiae, echymoses, and frank hemorrhage) were not observed in animals dying during the period of trauma or shortly thereafter. Splanchnic vascular engorgement was a common finding in control and hydralazine-treated animals which died early. However, no causal relationship between this vascular engorgement and acute traumatic death is apparent because splanchnic vascular engorgement was not found among the considerable number of Dibenzyline-treated animals which died during or shortly after the period of trauma.

Hydralazine was particularly effective in preventing early deaths in animals subjected to both levels of trauma tested. This protection logically may be attributed to an increased cardiac output and cerebral blood flow. Hydralazine is known to induce a marked and consistent increase in cardiac output in normovolemic animals and in man (11, 13, 14), and the drug also can increase cerebral blood flow (19, 20). The comparable protection provided by acute plasma volume expansion with PVP may be ascribed to a similar effect on cardiac output due to more adequate filling of the dilated vascular bed. In early experiments on drum shock, Noble and Collip (21) found no increase in susceptibility in rats bled 1 to 2 ml/100 g. However, they did not distinguish between early and late deaths, and some sensitization to the early effects of acute trauma might have been obscured.

If the above interpretation of the mechanism of early traumatic death as being due to acute hypotension and central nervous system ischemia is correct, the inhibition of vasomotor tone by Dibenzyline might be expected to increase the incidence of early deaths, as has been reported for large doses of ganglionic blocking agents (10). No increase in the incidence of early traumatic deaths among Dibenzyline-treated animals was noted in the present experiments. However, some increased sensitivity was suggested by the fact that a considerably higher percentage of the early deaths among Dibenzyline-treated animals occurred before the end of the drumming period, as had been noted in other studies with adrenergic blocking agents (22). In addition, Dibenzyline appeared to eliminate the protection against early deaths conferred by hydralazine or PVP in the absence of the adrenergic blocking agent. This effect appears to be explicable on the basis that increased cardiac output or intravascular volume

would be less effective in sustaining central nervous system circulation in the presence of a more dilated peripheral vascular bed.

The tendency of Dibenzyline to increase susceptibility to early traumatic death, the protection provided by hydralazine and PVP alone, but not in combination with Dibenzyline, and the increased volume of the liver and spleen during the drumming period in both Dibenzyline-treated and control animals all suggest an increase in vascular volume during the period of trauma. Hemodynamic studies made shortly after completion of a period of drum trauma (18) suggest that the venous bed is involved primarily, as reflected in a reduction in venous return with compensatory arteriolar constriction.

The almost complete absence of deaths among control animals in the period between 15 minutes and 2 hours after drumming suggests that two quite different mechanisms of death are involved, and the prominence of the gastrointestinal pathology characteristic of shock in animals dying in the late period indicates that these deaths are comparable to shock deaths induced by other procedures. The cause of death in "shock" has never been clearly established. However, such deaths characteristically are associated with some defect in body function which leads to death after a more or less protracted period even when the original shock-inducing stress has been fully corrected. This again is most clearly illustrated by hemorrhagic shock, where death usually occurs several hours after reinfusion of all shed blood.

In the present experiments the effects of drugs on the incidence of late, shock deaths were entirely different from those observed for early deaths. Hydralazine provided protection against shock deaths only when these were associated with minimal trauma. This limited protection appears to be most logically attributed to the vasodilatation which the drug induces, particularly in visceral vascular beds (12, 13, 14). It was considerably less than the protection provided by Dibenzyline, which effectively dilates vessels under sympathetic nervous system control, but which does not appear to have any direct effect on cardiac output. In the present experiments, Dibenzyline completely prevented shock deaths in all groups to which it was administered either alone or in combination with hydralazine or PVP. The effectiveness and essential irreversibility of the blockade produced by this agent (23) may explain the fact that in all of the combinations tested, the pattern of deaths was that characteristic of Dibenzyline treatment alone, and did not reflect the effects of concurrently administered hydralazine or PVP. The highly specific effect of Dibenzyline in preventing late, shock deaths suggests its use during the often protracted course of clinical shock.

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THE SILICA CONTENT OF GUINEA PIG TISSUES AS DETERMINED BY CHEMICAL AND ISOTOPIC TECHNIQUES[1]

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Abstract

The concentration of silica in guinea pig and cow tissues was determined by the silicomolybdic acid reaction. The silica concentration was relatively low in all tissues but was greater in hair and lung than in muscle, liver, and kidney. The silica concentration of tissues was apparently not influenced by the silica concentration of the diet. The silica concentration in guinea pig and cow tissues was approximately the same.

Experiments with $Si^{31}O_2$ indicated that orally administered silica was rapidly absorbed and excreted. The suggestion is made that silica is present both in an organic and inorganic form and that the latter is rapidly eliminated from the body.

Introduction

In a previous report (1) some aspects of silica metabolism in guinea pigs were studied in order to relate the findings to the problem of silica urolithiasis in Western range steers (2). It was noted that the excretion of silica in urine was related to the solubility rather than the quantity of silica ingested. In the present work the silica concentration in tissues was studied from animals fed low and high silica diets in order to determine if significant amounts of silica were stored in the body.

Gonnermann (3) found that the ash from various animal tissues contained approximately 2–10% SiO_2. King *et al.* (4, 5) used both colorimetric and gravimetric techniques for silica analyses and reported the following values expressed as mg SiO_2 per 100 g of dried tissue: *liver*, 12.0–30.6 (rabbit), 12.0–21.6 (ox), 4.4–10.0 (calf); *kidney*, 14.4–22.2 (rabbit), 7.8–9.6 (calf); *muscle*, 24.2–36.8 (calf); *lung*, 14.2–235.0 (rabbit), 81.2–120.0 (ox), 48.0–78.0 (guinea pig); *hair*, 15.6–16.5 (rabbit), 19.4–20.7 (guinea pig).

King (6) improved the colorimetric method for silica determination in organic matter by first removing phosphorus and iron from the sample. The silica content of defibrinated horse blood was 1.27–1.31 mg per 100 ml and that of oxalated human blood 1.14–1.66 mg per 100 ml. Holt (7) used this method and reported the following values for mouse tissue: *liver* 1–3, *spleen* 3–37, *kidney* 4–5, and *lymph glands* approximately 0.005 mg SiO_2 per 100 g of dried tissue.

King *et al.* (8) described a further modification of the method for the determination of silica in organic material. The silicomolybdate complex was reduced in 2 *N* sulphuric acid and under these conditions phosphorus did not interfere unless the concentration exceeded 4.8 mg per 100 ml of solution. Defibrinated horse blood was reported to contain 0.78 mg SiO_2 per 100 ml and heparinized human blood 0.19–0.37 mg SiO_2 per 100 ml. In the work reported here this method was used to measure amounts of silica of the order of 3–30 μg.

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The experiments with Si^{31} were carried out in order to determine absorption and excretion rates of orally administered silica sols and to compare the concentration of $Si^{31}O_2$ in tissues with the data obtained by chemical analyses.

Materials and Methods

Sodium carbonate was prepared by passing carbon dioxide through sodium ethylate which had been prepared from metallic sodium and ethanol. Commercial reagent-grade sodium carbonate was not suitable because of the presence of silica as a contaminant.

The tissues were dried for 24 hours at 110° C, finely ground in an iron mortar, and stored in polyethylene containers over calcium chloride until analyzed. The hair samples used for silica analysis (Table II) were first extracted for 48 hours with petroleum ether in a Soxhlet extraction apparatus in order to remove surface contamination. One gram of sodium carbonate was mixed with 1 g of tissue in a platinum crucible and partly ashed while exposed to air. The crucible was then covered with a loose-fitting lid and the ashing completed in a muffle furnace at 800° C for 2 hours. The mixture was fused for 1 hour over a Meker burner and the melt dissolved in distilled water. The silicomolybdate blue color was developed as described previously (1); however, in order to determine accurately the small amounts of silica present in tissues, polyethylene tubes were used instead of glass. The solutions were diluted 1:20 and absorbancy values determined on duplicate samples with a Beckman Model B spectrophotometer at a wavelength of 812 mμ in tubes with a light path of 25 mm. Values were read against sodium fluosilicate standards containing 1–30 μg SiO_2 in 25 ml water.

The Si^{31} used in these experiments was obtained by irradiating oscillator grade Brazilian quartz* in the Chalk River reactor for 8.0 hours at a maximum flux of about 9.5×10^{12} neutrons/cm^2/sec. Previous experiments had shown that commercial reagent-grade silica gel was unsuitable for this purpose because of the presence of sodium as a contaminant. The irradiated silica sample (300 mg) was obtained approximately 45 minutes after leaving the reactor and was immediately fused for 1 hour with 2 g of sodium carbonate. The cooled melt was dissolved in distilled water, partially neutralized with 1 *N* hydrochloric acid, and brought to volume in a 50-ml volumetric flask. The specific activity of the sample was determined by diluting an aliquot 1:2500 and counting it in an annular-type liquid Geiger tube (9). The decay curve of Si^{31} was used in extrapolating all results to zero time.

Male guinea pigs, raised from eighth generation inbred stock and weighing 492 ± 18.3 g† were dosed orally with 10-ml aliquots of the undiluted sols following a 24-hour starvation period. These sols were irradiated on separate days and had 25.8×10^6–32.1×10^6 c.p.m. per 10 ml at the time of dosing. The animals were placed in metabolism cages after dosing and were killed at intervals of 2, 4, 6, and 8 hours. Approximately 2 grams of liver, kidney, muscle, brain, and blood were weighed in tared glass tubes and digested with 10%

*Obtained through courtesy of the Department of Mines and Technical Surveys, Ottawa.
†Standard error of the mean.

alcoholic KOH in a boiling-water bath. The samples were brought to volume by diluting with distilled water and 10-ml aliquots removed for counting in an annular-type liquid Geiger tube (9). The bladder contents and excreted urine were pooled and counted directly after appropriate dilution. The dry weights of liver, kidney, and muscle were determined by drying for 24 hours at 110° C, after the Si^{31} had decayed. Brain and blood were not saved for dry weight determinations and the per cent dry weights were estimated from published values.

The Si^{31} content of the samples is reported as $Si^{31}O_2$ to facilitate comparison with data obtained by colorimetric analyses. All values were corrected for radioactive decay of Si^{31}.

Results

The recoveries of silica added to tissue samples are recorded in Table I. The results of the chemical analyses for the silica content of tissues are recorded in Table II. The tissues in experiment 1 were taken from 20 adult guinea pigs,

TABLE I

Recoveries of silica added to tissue samples

Tissue	μg SiO_2 added	μg SiO_2 recovered	% recovery
Liver	58	52	90
Lung	58	46	79
Lung	136	123	90
Heart	58	48	83
Heart	58	56	97
Muscle	36	35	97
Muscle	136	122	90

randomly divided into five groups of 4, and the tissues pooled for analysis. These animals were fed a diet of Purina guinea pig chow. The tissues in experiment 2 were obtained from four animals fed a soybean oil meal diet containing 0.75 mg SiO_2 per g of feed (1). The tissues were pooled for analysis. The tissues in experiment 3 were obtained from three animals fed a pelleted diet of Western hay containing 46.5 mg SiO_2 per g air-dried material (1). The tissues were pooled for analysis. Experiment 4 indicates the silica content of adult cow tissues obtained from a local slaughter house.

These results indicate that the silica content of tissues is relatively low and that with the exception of lung and hair it does not vary greatly, i.e. from 2.90 to 7.03 mg SiO_2 per 100 g of dried tissue.

The silica content of hair, in accordance with published results (5), is approximately 20 mg per 100 g. In the present work and in published results (4, 5) the silica content of lung is variable. This is probably due to the inhalation of silica-containing dust (10) and not to the accumulation of silica absorbed from the intestinal tract. These results indicate that the silica content of cow tissues is of a similar order of magnitude to that of guinea pig tissues. It therefore appears that the prolonged ingestion of silica-containing diets, such as hay, does not result in any appreciable storage of silica in tissues.

TABLE II

Silica content of animal tissues (mg SiO_2/100 g dry matter)

Expt. No.	Diet	Animal tissue	Liver	Kidney	Muscle	Blood	Heart	Lung	Hair
1	Guinea pig chow	Guinea pig (20)*	3.71 ± .290†	5.37 ± .855	7.03 ± .553	2.89‡ 3.77‡	5.84 ± .365	12.63 ± .181	21.28‡ 27.06‡
2	Soybean oil meal	Guinea pig (4)	5.65	4.13	5.05	3.77‡	5.84	5.47	
3	Western hay	Guinea pig (3)	3.83	4.68	4.92	—	4.92	8.94	34.6‡
4		Adult cow (2)	3.89‡	6.26‡	3.82	—	2.98	12.07	

*No. of animals.
†Standard error of the mean.
‡Analysis on tissue from one animal.

In the studies with Si^{31}, the samples were checked for radiochemical purity by half-life determinations (half-life of Si^{31} = 2.62 hours (11)). The irradiated sols, diluted 1:2500 and counted over a period of five or more half-lives showed no radiochemical impurity with the counting methods used in these experiments (Fig. 1). The Si^{31} in the urine samples, on the basis of half-life determination, was also radiochemically pure.

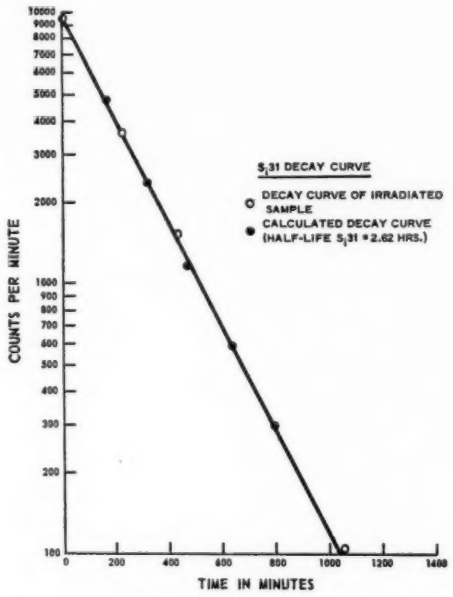

Fig. 1

In preliminary studies reagent-grade silica gel was irradiated to Si^{31} and administered orally to guinea pigs. It was noted that the tissues from these animals retained a radioactive impurity, tentatively identified as Na^{24}, in amounts sufficient to prevent an accurate estimation of Si^{31} content. The irradiated quartz that was used in the experiments reported here appeared to be radiochemically pure, and no attempts were made to identify impurities. The 4.7-hour tissue samples recorded in Table V were counted over a period of five Si^{31} half-lives and showed no evidence of a radioactive contaminant. However, it was noted that the radioactivity of the tissue samples with a relatively low Si^{31} content (Tables III and IV) remained approximately 30 c.p.m. above background after the Si^{31} had decayed. This represented a small amount of radioactive impurity that was retained in the tissues and had a half-life longer than that of Si^{31}. The results indicated that this impurity was distributed in the tissues independently of the Si^{31}. It therefore could have introduced an error in the tissue samples with a low Si^{31} content particularly if the Si^{31} had decayed for several half-life periods before counting.

TABLE III

Microgram $Si^{31}O_2$/g tissue (fresh wt.) in guinea pigs dosed with 10 ml of 0.6% labeled silica sol (pH 10.6)

Time (hr)	Liver	Kidney	Muscle	Brain	Blood	Urine (total $Si^{31}O_2$ excretion)
2	3.4 (2)*	8.8	2.0	0.6	4.2	1114.7
4	1.4 (2)	2.7	0.8	0.6	1.5	1354.7
6	1.7 (2)	2.9	1.5	1.1	1.9	2417.0

*No. of animals.

TABLE IV

Concentration of $Si^{31}O_2$ in tissues of guinea pigs dosed with 10 ml of 0.6% labeled silica sol (pH 11.8)
(A) μg/g fresh wt. (B) mg/100 g dry wt.

Time (hr)	Liver	Kidney	Muscle	Brain	Blood	Urine (total $Si^{31}O_2$ excretion)
4	(A) 3.1	6.5	2.3	1.3	3.7	4182.3
	(B) 1.1	2.8	0.9	0.5	1.8	
6	(A) 1.9	4.3	1.8	1.1	1.2	3016.7
	(B) 0.6	1.9	0.7	0.5	0.6	
8	(A) 2.2	3.8	2.0	1.4	2.0	4033.2
	(B) 0.7	1.7	—	0.6	1.0	

A quantitative determination of the size of the error contributed by the impurity was not attempted. It was probably small since the radioactivity of the tissue samples was relatively high. The error introduced by the impurity was greatest at 6 hours (Table III) and 8 hours (Table IV) because the Si^{31} decay with time was then greatest and a large factor was necessary to correct for it. No correction was made for the impurity, and since it had decayed proportionately less than the Si^{31}, its contribution to the recorded values was exaggerated when the radioactivity of the samples was extrapolated to zero time.

The results recorded in Table III are the mean values from two experiments carried out on separate days. Six guinea pigs were dosed orally with 10 ml of a 0.6% $Si^{31}O_2$ sol. This sol was adjusted to pH 10.6 prior to dosing by the addition of dilute hydrochloric acid which was added slowly with constant agitation to prevent the silica from gelling. The Si^{31} content in tissues was greatest at 2 hours, decreased by approximately half at 4 hours, and increased slightly at 6 hours. This small increase may have been the result of the impurity discussed above. The labeled silica in urine increased from 1.1 mg at 2 hours to 2.4 mg at 6 hours.

Table IV shows the results of orally dosing three guinea pigs with 10 ml of a 0.6% $Si^{31}O_2$ sol. The sol was adjusted to pH 11.4 with dilute hydrochloric acid prior to oral dosing. The results of this experiment are recorded in μg $Si^{31}O_2$ per g of fresh tissue and in mg $Si^{31}O_2$ per 100 g of dry tissue to permit comparison with the data obtained by chemical analyses. The labeled silica concentration of the tissues was maximum at 4 hours, decreased at 6 hours, and showed a small increase at 8 hours. This 8-hour increase may have been the result of the

above-mentioned impurity. The 4-hour values recorded in this table were approximately two to three times greater than the 4-hour values recorded in Table III. The labeled silica in urine was 4.2 mg at 4 hours and did not increase at 6 and 8 hours.

Table V shows the results of orally dosing three guinea pigs with 10 ml of sodium silicate solution containing 0.6% $Si^{31}O_2$. This solution was highly

TABLE V

Concentration of $Si^{31}O_2$ in tissues of guinea pigs dosed with 10 ml sodium silicate solution containing 0.6% $Si^{31}O_2$ (A) μg/g fresh wt. (B) mg/100 g dry wt.

Time (hr)	Liver	Kidney	Muscle	Brain	Blood	Urine (total $Si^{31}O_2$ excretion)
2	(A) 67.7	242.6	15.8	5.1	68.1	47.6
	(B) 19.7	105.6	4.8	2.1	32.4	
4.7	(A) 33.0	605.0	17.1	4.7	35.2	1806.6
	(B) 10.9	295.5	6.4	1.9	16.8	

alkaline because no hydrochloric acid was added before dosing and it therefore contained the sodium carbonate that was present in excess of that required for fusion. The tissue $Si^{31}O_2$ concentrations showed severalfold increases over those recorded in Tables III and IV. The urine $Si^{31}O_2$ excretion was low. At 4.7 hours the labeled silica concentration of liver and blood dropped to approximately half the 2-hour concentration but muscle and brain remained approximately the same. The concentration of silica in kidney increased to over twice the 2-hour level and was approximately 200-fold greater than the 4-hour level recorded in Table III. The urine $Si^{31}O_2$ excretion increased to the 4-hour value recorded in Table III but was well below the 4-hour value recorded in Table IV.

Discussion

In this study it was noted that the silica content of tissues, with the exception of hair and lung, was lower than earlier work indicated (4, 5). Silica values ranged from 2.98 mg per 100 g of dry weight tissue for cow heart to 7.03 mg per 100 g of dried guinea pig muscle. There were small but definite differences in the silica content of tissues. That, as well as the fact that hair contained relatively large amounts of silica may indicate the presence of organically bound silica as suggested by some workers (12, 13, 14, 15). The interpretation of the results for the silica content of lung is difficult because part of this silica is inhaled dust (10) and these data therefore do not necessarily reflect the 'endogenous' silica content of lung tissue. It is likely that kidney tissue may also have given erroneously high values because of the presence of silica in tubular fluid. The experiments with labeled silica sol (Tables III and IV) show that initially the rate of silica absorption is rapid but that it slows considerably after 4 hours. In an earlier report (1) the suggestion was made that silica absorption and excretion are primarily dependent on the solubility of the ingested silica. In these experiments the initial high rate of absorption

and excretion was probably due to the presence of silicic acid monomer and possibly silicate ion in the freshly prepared sol. The decreased rate of absorption may be related to the polymerization of the sol with aging in the presence of acid gastric juice. These data appear to be in agreement with a previous report (1) which showed that the silica excretion in urine did not exceed 10.3 mg in 48 hours when 80 mg of SiO_2 was administered orally as sodium silicate. The pH of the sol appears to be an important factor in regulating the absorption and excretion of ingested $Si^{31}O_2$. The results in Tables III and IV indicated that increasing the pH of the sol from 10.6 to 11.4 resulted in a threefold increase in the labeled silica contents of tissues and urine at 4 hours. Since the 6-hour values were approximately the same, it would appear that the pH effect on the solubility of ingested sols is of relatively short duration. These results are similar to the observation made by Holt *et al.* (16), who noted that Si^{31} tissue levels were greater with intraperitoneally injected sols of pH 10–12 than with sols of pH 5.5–7.

Table V shows the results of administering a solution of sodium silicate and sodium carbonate. The concentration of $Si^{31}O_2$ in the tissues was much greater than that recorded in Tables III and IV and was of a similar order of magnitude to that recorded by Holt *et al.* (15) after intraperitoneal injections of labeled silicon. These high values may have been caused by the absorption of silicate ions that are thought to be present above pH 10.9 (17) since it is unlikely that the gastric acidity was sufficient to neutralize this solution. The concentration of $Si^{31}O_2$ in the kidney at 4.7 hours was approximately three times greater than at 2 hours and approximately 200 times greater than found in the kidney at 4 hours in the experiments recorded in Table III. The reason for this large increase is not known but it may have been related to the accumulation of siliceous deposits in the renal tubules previously observed (18) when large quantities of a soluble silica (tetraethyl orthosilicate) were administered orally to guinea pigs. The formation of such deposits may also have been a factor in the low urinary excretion of labeled silica observed at 2 hours.

The presence of organically bound silicon in animal tissues has been reported (12, 13, 14, 15) although no evidence for a specific metabolic function of silicon was noted (15). In these studies the labeled silica in tissues reached a peak at 2 hours, then dropped to approximately half this value at 4 and 6 hours. The tissue silica concentrations obtained with the isotope, with the exception of the experiment in which a strongly alkaline sol was used (Table V), were considerably lower than those obtained by chemical analyses. It was also noted that the labeled silica concentration of liver was always greater than that of muscle, while this was not the case in tissues analyzed chemically for total silica.

These results suggest the possibility that two forms of silicon may be present in the animal body. The one form, determined by tracer experiments, is rapidly equilibrated with tissue fluids and excreted in the urine. The other form, comprising the greater part of the total silica determined by chemical methods, may represent an organically bound form of silicon that more accurately reflects the concentration of silicon in tissue.

Acknowledgments

The authors wish to express their thanks to Dr. A. M. Moore of the Biology Branch, Atomic Energy of Canada Limited, for his co-operation in making this work possible, to the staff of the Small Animal Laboratory for supplying the experimental animals, and to Mr. R. L. Brien for technical assistance.

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ISOLATION OF RAT HEPARIN[1]

L. B. JAQUES

Abstract

Heparin was isolated from the whole carcass and skin of rats of the Wistar strain and crystallized as the barium salt. The sodium salt derived from this had a specific anticoagulant activity (Howell assay) of 47.5 International Units/mg and specific metachromatic activity of 101.5 International Units/mg. Yield of heparin was 0.55 mg/100 g body weight.

Jaques, Charles, and Waters (1) demonstrated that when heparin is isolated as a crystalline barium salt from various species there are significant differences in the chemical and biological properties of the heparins so obtained. There is a difference in solubility of the barium salt and in particular there is a marked difference in the specific anticoagulant activity. The relative anticoagulant activities of dog, beef, pork, and sheep heparin were in the ratio of 10:5:2:1. Recently a number of investigators have carried out studies implying determinations of heparin, using rats. This is a particularly useful species from the standpoint of the mast cells. As these are present in large numbers in rat subcutaneous tissue, it is relatively easy, after subjecting animals to various procedures which cause changes in these cells, to study simultaneously changes in mast cells and tissue constituents. Before such figures can be meaningful for heparin, however, it is essential that rat heparin be isolated and characterized. After a number of attempts were made, the material was successfully isolated from rats using in principle the method of Charles and Scott (2). The isolation of heparin from the whole animal and from rat skin is described. Extraction of rat carcasses after removal of the skin failed to yield any active material, indicating that skin and subcutaneous tissue constitute the main depot for heparin in the rat.

Experimental

The extraction and further purification of heparin was carried out as described by Jaques and Bell (3, pp. 267, 268). The heparin present was assayed by comparing its anticoagulant activity on fresh cat blood (3, p. 283) and its metachromatic activity on Azure A (3, p. 292) with that of a sample of the International Standard of Heparin.

Lot 1

Forty-one very old rats were killed by stunning, eviscerated, the skins were removed, and the whole rats minced. Weight of rat mince was 11.2 kg. The minced tissue was extracted with alkaline ammonium sulphate (8.3%) solution at 50° C and the extract precipitated at pH 2.5. After extraction with alcohol, the precipitate was digested with trypsin. After precipitation with acid alcohol,

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Contribution from the Department of Physiology and Pharmacology, University of Saskatchewan, Saskatoon, Saskatchewan. This work was supported by a grant from the National Research Council of Canada.

and extraction with hot acetone, the crude heparin was obtained as a dry powder. Weight, 112.0 g. Metachromatic activity, 1.7 I.U./mg.

The crude heparin was treated with 10% ammonium carbonate at 70° C and the clear filtrate was acidified and then precipitated with alcohol. Weight, 5.89 g. Metachromatic activity, 11.3 I.U./mg. This was dissolved in 20 ml of water and again treated with 10% ammonium carbonate. The solution was then acidified, treated twice with charcoal, dialyzed for 36 hours, and excess benzidine added. The precipitate of benzidine–heparin was hydrolyzed with 5 *N* NH_4OH, centrifuged, and the supernatant treated three times with Lloyd's reagent with addition of acid. The heparin was then precipitated with alcohol. Weight, 208.5 mg. This was dissolved in 2.5 ml of water, treated with ammonium carbonate to remove calcium, and then precipitated with 9 volumes glacial acetic acid. Weight, 147.5 mg. This was dissolved in 1 ml of water with 0.2 ml of 5 *N* NH_4OH and a trial crystallization of the barium salt was carried out with 0.05 ml. Good crystallization was observed in the gross and, microscopically, there were uniform, very small crystals. To the remaining (0.9 ml) solution were added 2.25 ml of water, 0.75 ml 10% barium acetate, and 0.685 ml glacial acetic acid. This gave a good crystallization in small rosettes and sheaves which were uniform in size. These were centrifuged, washed, and dried. Weight of barium salt, 70.7 mg. Metachromatic activity, 81 I.U./mg. Anticoagulant activity, 45 I.U./mg. Yield, 2.3 mg/kg.

Of the barium salt 3.5 mg was kept for assay and the remainder converted to the sodium salt. For this, the barium was removed with ammonium carbonate, the carbonate with acetic acid, and the solution adjusted to pH 7.0 with 5 *N* NaOH and acetic acid and precipitated with three volumes of MeOH. There was some difficulty in obtaining a clear supernatant after ammonium carbonate as a sludge formed. Weight of sodium salt, 54.6 mg. Metachromatic activity, 88 I.U./mg. Anticoagulant activity, 50 I.U./mg.

Lot 2

After 261 rats were stunned and decapitated, the hair was removed with clippers, the animals were skinned, and the heads and tails were discarded. Each rat's carcass was minced and batches of the mince combined and stored in the deep-freeze until all the animals had been sacrificed (over a 2-day period). The skins were similarly minced and stored in the deep-freeze.

Lot 2A. Extraction of Rat Carcass for Heparin

The combined carcasses weighed 52 kg. These were allowed to stand overnight to thaw and then allowed to autolyze at room temperature for 6 hours. The tissue was extracted with alkaline ammonium sulphate (8.3%) solution at 50° C. The extract was precipitated at pH 2.5, and after extraction with alcohol, the precipitate was digested with trypsin. The digest was precipitated with acid alcohol. After the precipitate was extracted with hot acetone, the resulting crude heparin powder weighed 744 g and showed no metachromatic or anticoagulant activity. This was redigested with trypsin. Weight, 632 g. The crude material was heated in 10% ammonium carbonate at 55° C and filtered. The filtrate was acidified and precipitated with acid alcohol. The

precipitate contained a large mass of white crystals. Weight, 158 g. Metachromatic activity, 1 I.U./mg. Anticoagulant activity, < 1 I.U./mg. A series of experiments were made to purify this material by fractionation with alcohol, cold, etc. No increase in the specific metachromatic activity of the fractions was found.

Lot 2B. Extraction of Heparin from Rat Skin

The total weight of the hides was 9.86 kg. These were removed from the deep-freeze and allowed to stand overnight at room temperature. At the end of this time they were not completely thawed. They were extracted with alkaline ammonium sulphate (8.3%) solution at 50° C. The extract was precipitated at pH 2.5. Considerable difficulty due to collagen was encountered in filtration at this stage. The precipitate was extracted with alcohol and digested with trypsin. The digest was precipitated with acid alcohol and after extraction with hot acetone, a crude powder was obtained. Weight, 22.1 g. Metachromatic activity, 5.7 I.U./mg. This crude precipitate was dissolved and dialyzed against tap water for 5 hours. The solution was then reduced in volume, dialyzed against distilled water, and further reduced in volume to 75 ml. Ammonium carbonate was added, the mixture heated to 70° C and filtered. The filtrate was acidified with glacial acetic acid and excess benzidine added. The benzidine precipitate was hydrolyzed with 5 *N* NH_4OH, the benzidine removed in the cold, and the filtrate treated with Lloyd's reagent. After a second treatment with Lloyd's reagent at pH 6, the filtrate was precipitated with an equal volume of 95% alcohol. Weight of precipitate, 750 mg. The precipitate was dissolved in alkaline water, treated with ammonium carbonate, and then precipitated with 9 volumes of glacial acetic acid. Weight, 633.5 mg. This was dissolved for trial crystallizations. The barium salt crystallized out in typical rosettes and sheaves. The solution remaining after trials was made up to a volume of 14.3 ml and 3.43 ml of 10% barium acetate and 4.00 ml glacial acetic acid added at 65° C. On cooling, the barium salt crystallized out in typical rosettes and sheaves. Weight, 482 mg. The barium salt was converted into the neutral sodium salt by removing the barium with ammonium carbonate, removing the carbonate with acetic acid, and finally neutralizing with 5 *N* NaOH and precipitating with 3 volumes of 95% methyl alcohol. Weight of sodium salt, 341 mg. Metachromatic activity, 101.5 I.U./mg. Anticoagulant activity, 47.5 units/mg. Yield = 5.5 mg/kg of body weight or 33.5 mg/kg of skin.

Analysis of sodium salt 2B: C, 21.55%; H, 5.34%; N, 5.91%; S, 11.27%; acetyl, 8.06%; ash (sulphated), 11.6%; water, 14.3%; $[\alpha]_D^{16} = +44.5°$. Anticoagulant activity (fresh cat blood) 46.6, 46.7, 48.2; average 47.5 I.U./mg. Metachromatic activity (Lovibond) (mean of 17 readings) 101.5 (S.D. 7.38) I.U./mg; U.S.P. assay, 57.8% of International Standard = 75 units/mg. (Commercial sample of beef heparin, 61.2% = 80 units/mg.) Potency calculated for corresponding anhydrous free acid: anticoagulant activity 57.8 I.U./mg; metachromatic activity 123 I.U./mg.

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UREA PRODUCTION IN CHICK LIVER SLICES[1]

ANDRÉ LEMONDE[2]

Abstract

Liver slices of very young white Leghorn chicks have been found capable of urea production. A correlation seems to exist between the protein level of the diet and the urea formation in the liver. When birds are subjected to a relatively long fasting period, they metabolize their own tissue proteins, and urea appears in the liver.

Studying the synthesis of urea in rat liver slices, Krebs and Henseleit (1) have found that relatively little was formed in the livers from starved animals. Supplying oxygen to these livers was not sufficient to improve ureogenesis completely. It was necessary to add minute amounts of ornithine or citrulline to restore the urea synthesis entirely. On the basis of these findings and from the known distribution of the enzyme arginase in liver (2), these authors proposed the ornithine cycle.

In their paper, Krebs and Henseleit (1) reproduced the data obtained by Edlbacher and Röthler (2) on the arginase content of different animal tissues. The latter authors have demonstrated the almost complete absence of arginase in the fowl liver. Knowing that birds have a uricotelic metabolism, it was logically concluded that the bird liver cannot produce urea.

The results described in this paper show that under certain circumstances chick liver slices can produce urea.

Experimental

One-day-old white Leghorn cockerels were obtained from a poultry farm. They were fed a commercial diet. Feeding was generally stopped 24 hours before a given experiment. Fasting birds were supplied with water ad libitum.

The chicks were killed by decapitation and the livers were rapidly removed and suspended in a chilled washing solution containing 1000 ml of NaCl 0.9%, 20 ml of KCl 1.15%, and 20 ml of $CaCl_2$ 1.22%.

In all experiments, the tissue-slice technique was employed, adopting the procedure described by Gornall and Hunter (3) with only minor modifications. The livers were cut freehand with a thin razor blade, and slices were washed thrice in the washing solution. A number of slices, estimated to have a total dry weight of about 10 mg, were distributed in Warburg vessels containing the required reaction mixture. The flasks were filled with the gas (95% O_2 and 5% CO_2) and incubated at 37° C for 2 hours. After the incubation, the liver slices were removed from each vessel, dried, and weighed.

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Contribution from the Department of Biochemistry, School of Medicine, Laval University, Quebec, Que. This work was supported by a grant from the National Research Council of Canada.

[2]Holder of a Medical Research Associateship from the National Research Council of Canada.

The reaction mixture consisted of the physiological solution Krebs–Ringer–bicarbonate (4) to which were added 0.015% of ammonia in the form of an 0.827% ammonium chloride solution and 0.2% of lactate in the form of a 3% solution of sodium lactate. A complete description of this reaction mixture is given in Table I.

TABLE I

Sample protocol

NH_4Cl 0.827% (NH_3: 0.015% of the reaction mixture)	0.18 ml
Na lactate 3% (lactate: 0.2% of the reaction mixture)*	0.21 ml
Krebs–Ringer–bicarbonate (4)	2.81 ml
Acetate buffer (pH 5)	0.30 ml
Urease (Jack bean)†	0.50 ml
Final volume	4.00 ml
Dry weight of slices	10.00 mg
Urea in 2 hours (expressed in μl of CO_2)	65.60
Urea quotient‡	2.10

*Crystallized lactic acid neutralized with NaOH to pH 7.4 and diluted to give a 3% solution of Na lactate.
†From the Arlington Chemical Company, New York.
‡$\frac{\mu l \text{ of } CO_2 \text{ from urea}}{(\text{mg of dry slices in a vessel}) \times (\text{hours of incubation})}$.

Urea determinations in the residual medium, after the removal of liver slices, were carried out by the manometric urease method of Krebs and Henseleit (1). The results are expressed in terms of the urea quotient (μl of CO_2 from urea per mg of dry liver per hour). The sample protocol in Table I will clarify the procedure.

Results and Discussion

The results assembled in Table II show that the liver slices of very young chicks are capable of urea production. As the chick grows, urea formation becomes less significant and disappears almost completely at the age of 30 days.

TABLE II

Urea formation in chick liver slices

Age in days	Number of observations	Urea quotient	
		Mean $\pm \sigma M$*	Maximum deviation
1	27	1.10±0.040	0.26–2.22
2	56	1.27±0.056	0.57–2.22
3	55	0.80±0.016	0.28–2.87
4	54	0.94±0.017	0.21–1.75
5	54	0.84±0.020	0.20–1.92
6	27	0.50±0.049	0.12–1.13
7	56	0.61±0.034	0.18–1.38
10	56	0.71±0.060	0.06–1.83
15	12	0.61±0.100	0.12–1.08
20	13	0.61±0.087	0.00–1.08
30	12	0.13±0.048	0.00–0.48

$\sigma_M = \sqrt{(m-M)^2/N(N-1)}$, standard error of the mean

On account of these data, we attempted to explain this urea formation in the chick liver. Bauerova and Sorm (5), in their study on the arginase activity in

liver of birds, have found considerable differences among the species studied. "Corvidae and the kestrel have very active arginase in the liver, while in wild Galliformes and domestic fowl, the arginase activity in the liver is very much less. In domestic fowl, the maximum arginase activity is found in the liver of the chick. The arginase activity in the livers of Galliformes increases on starvation".

On the basis of their results, Bauerova and Sorm (5) concluded that the arginase activity in bird liver homogenates depends on the protein content of the diet. Corvidae and the kestrel live on a diet rich in protein and have a high arginase activity in the liver. Domestic fowl, fed a diet rich in carbohydrates but low in proteins, show low arginase activity in their livers but, after a certain period of fasting, the birds began to metabolize their own proteins causing arginase activity to increase.

Such a variation of arginase activity in the livers of animals receiving a high or low percentage of proteins in their diets was observed by many authors (6, 7, 8, 9, 10). Knox, Auerbach, and Lin (11) have recently reviewed these enzymatic and metabolic adaptations in animals. For the special case of arginase activity, they wrote that the factor responsible for these changes is not clearly known.

This relation which seems to exist between the protein content of the diet and the arginase activity might explain our observed urea formation in chick livers. During the days after hatching, a 24-hour fast is sufficient to increase the arginase activity. The more the birds grow, the longer the fasting period has to be for a similar arginase activity to be attained.

In order to prove this idea, we measured the urea formation in liver slices of 6- to 8-week-old white Leghorn cocks. The fasting period varied from 0 to 7 days. The data given in Table III demonstrate beyond doubt the effect of

TABLE III

Effect of starvation on urea formation in chick liver slices*

Number of observations	Days of starvation	Urea quotient	
		Mean $\pm \sigma M$	Maximum deviation
15	2	0.04	0.00–0.40
13	3	0.13 ± 0.042	0.00–0.56
14	5	0.30 ± 0.100	0.00–1.09
27	6	1.50 ± 0.208	0.15–3.97
17	7	1.59 ± 0.270	0.36–3.72

*Age of chicks, 6 to 8 weeks.

starvation on urea production in chick liver slices. Moreover, these figures obtained with liver slices are in complete agreement with those published by Bauerova and Sorm (5), who used tissue homogenates. Lightbody and Kleinman (12) have also observed such an increase in liver arginase activity of animals submitted to a severe starvation.

From these data, it is evident that the liver of very young chicks can produce urea. We have also shown that under severe conditions of starvation the liver again can produce urea. In order to elucidate the mechanism of this urea

formation, we have made a number of measurements in an ammonia-free medium. The results indicate the same urea production in the absence of ammonia. The urea found in the liver slices of chick is thus not synthesized from ammonia. It could be simply produced by the action of a small amount of arginase on arginine.

Acknowledgments

The technical assistance of Miss Colette Methot is gratefully acknowledged. The author is indebted to Fred W. Bray, Limited, Hamilton, Ontario, for supplying all the cocks used in this research. The author also acknowledges the valuable suggestions of Dr. Fernand Martel, Department of Biochemistry, Laval University.

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THE EFFECTS OF DIFFERENT VARIETIES OF RAPESEED OIL ON WEIGHT GAIN, AND OF GOLDEN RAPESEED OIL ON REPRODUCTION, OF THE RAT[1]

JOYCE L. BEARE, E. R. W. GREGORY, AND J. A. CAMPBELL

Abstract

Rapeseed oil of the Polish, Golden, or Swedish varieties, corn oil, or mixtures of corn oil and ethyl erucate were fed to weanling rats of the Wistar strain for 9 weeks. Weight gains were inversely related to the content of erucic acid in the diet, confirming previous indications that the growth retarding effect of rapeseed oil is due to its erucic acid. In general, the differences in weight gains could be explained by the effect of rapeseed oil on food consumption.

Golden rapeseed oil and corn oil were compared in a reproduction study involving three litters of one generation. Although the young of rats fed rapeseed oil were of lesser weanling weight, there was no difference in the number of animals successfully weaned.

Introduction

It was shown by Craig (1) that oils from various types of rapeseed differed in their composition of fatty acids, particularly with respect to erucic acid. Thomasson and Boldingh (2) demonstrated in rats that nasturtium seed oil with more erucic acid than rapeseed oil was a greater inhibitor of growth, and that the addition of erucic acid to a diet decreased its growth-promoting qualities. The effect on rats of different varieties of rapeseed oil and of mixtures of ethyl erucate and corn oil have, therefore, been studied.

In view of the report of Carroll that dietary erucic acid caused an impairment of spermatogenesis, and might be useful in controlling fertility (3), it was of interest to investigate further the effect on reproduction of a diet containing erucic acid. The performance of rats receiving rapeseed oil and corn oil were compared during the production of three litters.

Methods

One hundred and twelve weanling, inbred rats of the Wistar strain, of equal sex distribution, were arranged in a randomized block design according to sex and initial weights, and housed in individual, screen-bottomed cages. Each of the eight rats in a block received a different dietary oil. Corn oil, crude Golden[2] rapeseed oil, refined Swedish, Golden, and Polish rapeseed oils, a mixture of 51% ethyl erucate and 49% corn oil, and a mixture of 26% ethyl erucate and 74% corn oil were added at the level of 20% by weight to the purified basal diet previously described (4). Food and water were supplied ad libitum. The body weight and food consumption of each rat was recorded twice weekly.

After 9 weeks on experiment, all animals were killed with the exception of seven males and seven females fed the diet containing the refined Golden

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Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Ontario.

[2]Golden rapeseed is a strain derived from a selection of Canadian-grown Argentine rapeseed.

rapeseed oil, and the same number of each sex fed the diet containing corn oil. A comparable control group was fed a commercial fox diet.[3] Vaginal smears were studied for 21 days prior to the first matings when females approximately 110 days of age were bred with males of the same age supplied with the same diet. On the third day after parturition, the pups were counted and then left undisturbed, except for a cage cleaning after the 10th day, until they were weaned when they were 21 days of age and their body weights recorded. After the parents had produced three litters, they were killed when approximately 46 weeks of age and examined for gross pathology. The adrenals and testes were removed and weighed.

Results and Discussion

As shown in Table I, the weight gains of rapeseed oil fed rats after 3, 6, and 9 weeks were inversely related to the content of C_{22} fatty acids, essentially

TABLE I

Compositions and weight gains obtained with three varieties of rapeseed oil

	Fatty acid composition,* %				Weight gains, † g					
					Males			Females		
Variety	C16	C18	C20	C22	3 wk	6 wk	9 wk	3 wk	6 wk	9 wk
Polish	3.5	51.1	16.5	28.9	64±2	129±5	176±6	57±2	87±4	110±3
Golden	4.4	37.2	15.7	42.7	49±2	103±7	146±8	36±2	70±3	95±4
Swedish	3.8	35.8	15.9	44.5	47±2	101±4	143±6	37±3	74±4	91±4

*Obtained by gas phase chromatography through the courtesy of Dr. B. M. Craig.
†Mean weight gain ± standard error.

erucic. Polish rapeseed oil, containing the lowest level of erucic acid, promoted the greatest weight gains. Golden and Swedish rapeseed oils which were similar in C_{22} content produced similar weight gains. These results support the conclusion of Thomasson that the erucic acid content of rapeseed oil is responsible for the smaller weight gains of rats fed the oil (2).

Total food consumption and weight gains obtained with each diet for the 9-week experimental period are shown in Fig. 1. The data on male rats were separated into three distinct groups with respect to both food consumptions and weight gains; corn oil and 26% ethyl erucate in corn oil were significantly higher ($P=0.01$) than 51% ethyl erucate in corn oil and Polish rapeseed oil, which were, in turn, higher than crude and refined Golden, and Swedish rapeseed oils. When the weight gains were adjusted to the same food consumption by a covariance analysis, all significant differences disappeared. For the females, the consumption of the diet containing 26% ethyl erucate in corn oil was significantly higher, and that of the diet containing refined Golden rapeseed oil significantly lower than that of any other diet. The weight gains of females receiving corn oil, 26% ethyl erucate in corn oil, and Polish rapeseed oil were similar, as were those of animals receiving Polish rapeseed oil, 51% ethyl

[3]Master Fox Cubes.

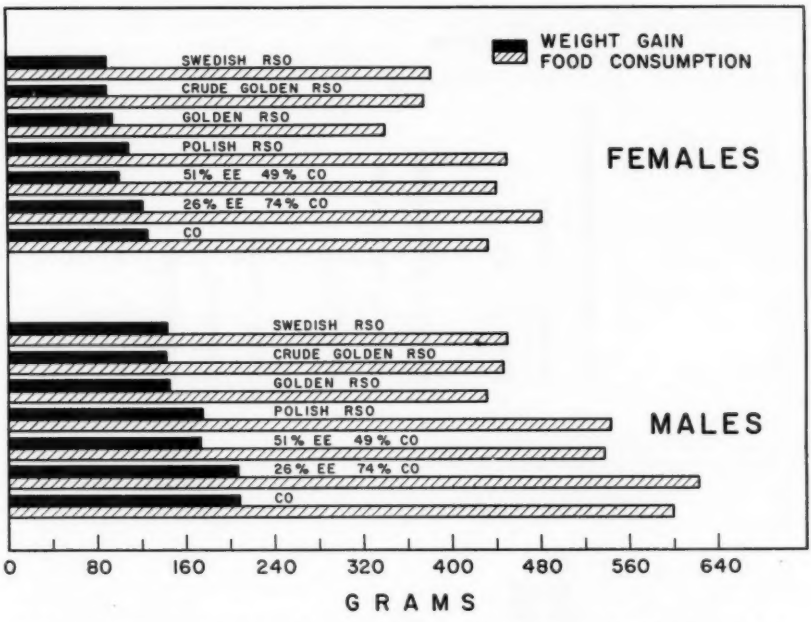

FIG. 1. Nine-week weight gains of rats fed Swedish, Golden, and Polish rapeseed oils (RSO), mixtures of ethyl erucate (EE) and corn oil (CO), and corn oil.

erucate in corn oil, and refined Golden rapeseed oil. The latter two dietary oils were not significantly different from crude Golden and refined Swedish rapeseed oils. When the weight gains were adjusted to the same food consumption by covariance analysis, only the lesser gains obtained with 51% ethyl erucate in corn oil could not be accounted for in terms of food consumption.

The females rats showed no irregularities in their estrus cycles that could be attributed to diet. During the production of three litters, the mean numbers of offspring born per female of the animals receiving the commercial diet and the purified diets containing corn oil and rapeseed oil were 24.9, 19.1, and 15.2 respectively. The corresponding numbers of offspring successfully weaned were 21.7, 11.0, and 13.3 per female. In Fig. 2 are shown the mean numbers of animals born and weaned per litter. It is seen that rats fed the commercial diet had a superior reproductive performance, particularly in the first litter. Statistical analyses revealed that differences between the diets containing corn oil and rapeseed oil were insignificant both with respect to the number of young born and the number weaned. The mean weanling weights of the pups of each litter are shown in Fig. 3. Fox cubes and the purified diet containing corn oil were not significantly different in their effect on weanling weight. With rapeseed oil in the parental diet the weight of the weanling rats was consistently lower. This fact might be related to a decreased milk production resulting from the reduced food intake of animals receiving rapeseed oil and

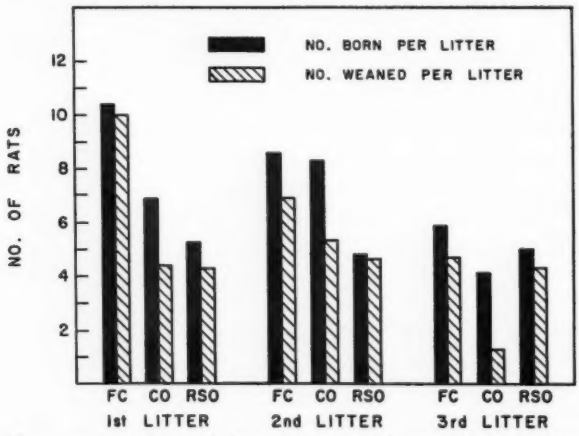

FIG. 2. Mean number of animals born and weaned in each of three litters of rats fed fox cubes and purified diets containing 20% corn oil or rapeseed oil.

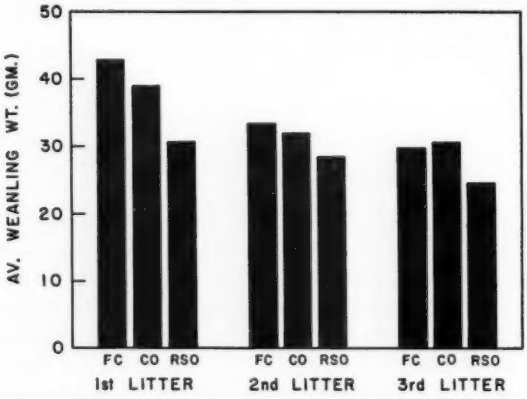

FIG. 3. Mean weanling weights of pups of rats fed fox cubes, 20% corn oil with a basal purified diet, and 20% rapeseed oil with a purified basal diet.

to the presence in the milk of some erucic acid. Hilditch and Thompson (5) found small amounts of erucic acid in the milk of cows.

After the rats had produced three litters and when they were approximately 46 weeks of age, differences in body weights of those animals fed corn oil and rapeseed oil were no longer significant. Testes and adrenal weights of the animals fed the two oils also showed no significant differences.

Carroll and Noble (3) concluded that 10% or more erucic acid added to a powdered commercial meal impaired the spermatogenesis of Sprague–Dawley rats and within 5 months produced sterility in them. In the study reported here, the males of Wistar rats fed 20% Golden rapeseed oil, equivalent to 8.5% erucic acid, successfully sired three litters, and showed no abnormality at approximately 11 months of age. It may also be noted that rapeseed oil

contains a substantial fraction of linoleic acid and a somewhat lower level of erucic acid than that employed in the studies of Carroll and Noble in which erucic acid was the only fatty acid added to a low fat meal. The results of the present study fail to support the theory that erucic acid induces sterility in rats receiving an adequate diet.

Acknowledgments

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MACROMOLECULAR PROPERTIES OF VITELLENIN FROM EGG YOLK AND ITS PARENT COMPLEX WITH LIPIDS[1]

W. G. MARTIN, K. J. TURNER,[2] AND W. H. COOK

Abstract

The floating fraction of egg yolk (90% lipid, density 0.98) appears to be heterogeneous but no significant fractionation took place during centrifugation in several solvents of density 0.98 or higher. This fraction had a particle size several times that of the lipoprotein (50% lipid) remaining after ether extraction. Most of the so-called "free" lipid extracted by ether is apparently part of, or associated with, the lipoprotein.

The lipid-free vitellenin was not soluble in mild aqueous solvents and most of the physical measurements were made in 88% formic acid solutions. Molecular weights ranged from 3.0 to 9.0×10^4. The higher value, considered most reliable, was only half the estimated size of the protein part of the lipid-containing materials. Evidently the protein portion of the lipoprotein has two or more polypeptide chains that may be combined directly or through lipid by forces weaker than the peptide linkage.

Introduction

In an earlier paper (1) it was shown that the lipoprotein isolated from the low-density floating fraction (90% lipid) of egg yolk by ether extraction, and termed lipovitellenin by Fevold and Lausten (2), was probably derived from a natural entity of higher lipid content. About two-thirds of this lipoprotein was insoluble after ether extraction and this portion contained 43% lipid. The soluble material had 53% lipid, and was polydisperse and apparently heterogeneous in lipid content. Since lipovitellenin is evidently a derived product of intermediate lipid content, the entire floating fraction and lipid-free protein (vitellenin) derived from it were investigated.

The heterogeneity of the ether-extracted lipoprotein was attributed in part to variations in lipid content (1) but polydispersity arising from the protein moiety must also be considered. Measurement of the molecular weight of vitellenin was therefore made to assess the homogeneity of the protein moiety.

Materials and Methods

The floating lipid-protein fraction was separated from solutions of egg yolk as the supernatant layer on 2.5% sodium chloride solutions using the "layering" technique (1).

Initial preparations of lipid-free vitellenin were made by slowly adding the floating fraction in 2.5% sodium chloride solution at 0° C to 10 volumes of agitated chloroform–methanol (2:1) maintained at −23° C and stirring the resultant mixture for 2 hours at this temperature. Two volumes of ethyl ether were then added to facilitate decantation. This extraction–decantation pro-

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[2]National Research Council of Canada Postdoctorate Fellow 1956–58. Present address: Commonwealth Serum Laboratories, Parkville, Melbourne, Australia.

cedure was performed three times, and the protein washed three times with cold ether. The ether-saturated protein was then suspended in water at 0° C and dialyzed exhaustively against water at this temperature to remove residual lipid solvents. Subsequent lyophilization gave a finely divided dry product.

Preparations used for most of the quantitative work were made by a modification of this method when it was observed that complete removal of the mixed organic solvent at low temperature reduced the tendency of the protein to aggregate in formic acid. The initial steps were as described above but, instead of adding cold water, we removed the residual mixed organic solvent (3, 4) at −18° C by ether extraction in a jacketted Soxhlet apparatus. Sodium was placed in the boiling flask to remove alcohol, and extraction was continued for 20 hours. The product was then dried in vacuum at room temperature.

Unless stated otherwise, the formic acid used as solvent was 88% (w/w) and is subsequently referred to as formic acid. Mixtures with methanol were made on a volume basis and are reported as per cent methanol (v/v) in formic acid.

Most of the chemical and physical measurements were made by conventional methods previously described (5). Concentration was determined by dry weight or by specific refractive index increment calibrated by dry weight (5); measurement of density was made in a magnetic float apparatus (6); diffusion by integral fringe or schlieren methods (7); sedimentation in a Spinco Model E ultracentrifuge; osmotic pressure in Donnan and Rose osmometers (8); light scattering in a Brice–Speiser photometer (9); and viscosity in Ostwald-Fenske capillary viscometers.

Minor modification of conventional methods was required by the nature of the solute and the strong solvents necessary to disperse the protein after lipid removal. Although Visking dialysis tubing was weakened by formic acid at room temperature, it was found satisfactory for dialysis at 5° C. Collodion membranes were used for the osmotic pressure measurements with this solvent. Both dialysis and osmotic pressure measurements in dilute sodium hydroxide solutions were made in Visking tubing since alkali disintegrated collodion membranes. There was no evidence that either solvent dissolved the membranes used.

Since solutions of the floating fraction had a lower density than the solvent, the conventional U-cell of the Tiselius electrophoresis apparatus had to be inverted. This was done by adding ground glass ball-and-socket joints to the top sections of the cell and electrode vessels. The supporting cradle was modified to accommodate the U-cell and permit its manipulation in an inverted position. The connections between the inverted cell and electrode vessels were made with curved glass tubes fitted with ball-and-socket joints, and stopcocks for the manipulation of the boundaries. The cell was filled in the normal position, the curved tubes were attached, stoppered, and filled before the assembly was inverted for attachment of the electrode vessels. The relatively high conductivity of the solvent and the small density differential between solvent and solution necessitated low current densities (8 ma or less) to avoid boundary disturbances.

Results

Floating Lipid–Protein Fraction

The results of measurements on the entire floating fraction are summarized in Table I. From density determinations the partial specific volume v was found to be 1.025 ml/g at solvent densities of 1.014 and 1.030 g/ml. Accurate measurements were impossible at higher solvent densities. Partial specific volume was also estimated by extrapolating the $\eta_0 s_f^0$ vs. ρ_0 (1.014 to 1.080 g/ml) plot (Fig. 1) fitted by least squares, to $\eta_0 s_f^0 = 0$, where s_f^0 represents the negative

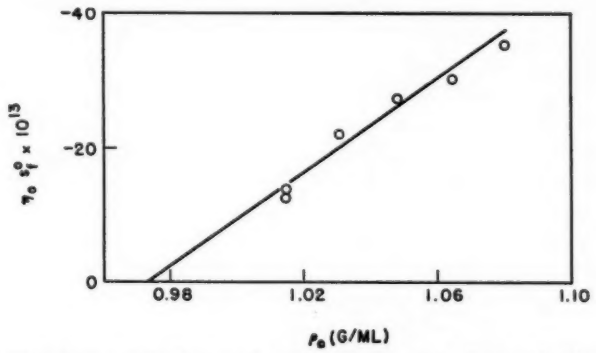

FIG. 1. Flotation rate ($\eta_0 s_f^0$) as a function of solvent (sodium chloride solutions) density.

sedimentation rate (flotation) extrapolated to zero concentration, and η_0 and ρ_0 represent the solvent viscosity in centipoises and density, respectively. This gave $\bar{v} = 1.028 \pm 0.007$ ml/g, a mean value in fortuitous agreement with that obtained from dry density, considering the standard error of the intercept.

With this information on specific volume, fractionation was first attempted at solvent densities of 1.01 to 1.40 g/ml. Some 20 solvents of different composition were used, the solutions centrifuged for various periods, and the supernatant and subnatant layers analyzed for lipid. There was no evidence that a useful degree of fractionation occurred.

Solvents having a density of 0.98 g/ml or lower were prepared using ammonium carbonate (0.5%) or glycine buffer (μ = 0.1 pH 9.0) to obtain the necessary solvent action with the minimum density increment and then adding a miscible low-density organic liquid. The addition of ethanol and acetonitrile (ca. 15%) caused no visible change in the solutions at 4° C. On centrifuging and analyzing these solutions, however, most of the fraction was found in the supernatant layers and any sedimenting material was an insoluble precipitate, indicating that some denaturation had occurred. The remaining solute apparently took up the light organic solvent, causing a density decrement that precluded fractionation.

Although these fractionation experiments were unsuccessful, the material was apparently heterogeneous. The patterns obtained from sedimentation (Fig. 2A) and electrophoretic (Fig. 2B) examinations do not demonstrate this

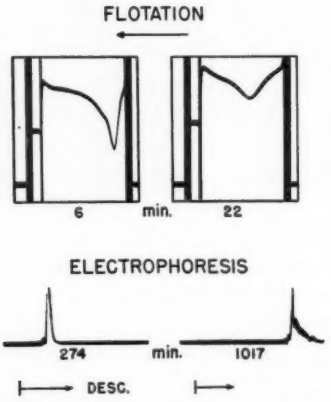

FIG. 2. Flotation and electrophoretic patterns of floating fraction. Flotation in sodium chloride 1.014; electrophoresis in 0.1 μ glycine buffer pH 9.0.

clearly, since the low mobility (main peak—2.4 to 2.8×10^{-5} $cm^2 volt^{-1} sec^{-1}$ in 0.1 μ glycine at pH 9.3) and density reduce the sensitivity of these methods. The shoulder on the leading edge of Fig. 2A suggests the presence of more than one component but the patterns must be interpreted with caution.

Estimates of particle size were obtained from intrinsic viscosity ($[\eta]$) and s_f^0 coefficients, since solutions of the complex could not be clarified sufficiently for light-scattering or diffusion measurements. The intrinsic viscosity (Table I) in

TABLE I

Summary of measurements on floating fraction

Property		Solvent density at 20° C (sodium chloride solutions)	Value
$dn/dc \times 10^3$ (5780 Å)	dl/g	1.014 and 1.030	1.52 ±0.02
$\bar{v}$ (magnetic float)	ml/g	1.014 and 1.030	1.025±0.002
$\bar{v}$ (Fig. 1)	ml/g	1.014 to 1.080	1.028
$[\eta]_{20}$	dl/g	1.014	0.028
		1.030	0.047
$s_{f\ 20,w}^0$ (Svedbergs)		1.064 and 1.080	7.5
$M_w \times 10^{-6}$ from s and $[\eta]$		As for $[\eta]$ and s_f^0	4.8

2.5% sodium chloride indicates an essentially spherical molecule. However, the experimental results are inadequate to determine whether the increase in $[\eta]$ in 5% sodium chloride is due to swelling, elongation, or aggregation.

The error in determining $\bar{v}$ introduces an uncertainty in the buoyancy factor $(1-\bar{v}\rho_0)$ that decreases as the solvent density is increased. The $s_{f\ 20,w}^0$ value reported was that obtained at the higher solvent densities, since the differential uptake of the components from the concentrated binary solvent was considered a secondary source of error. These $[\eta]$ and s_f^0 values were used to estimate M_w by the Scheraga–Mandelkern equation (10):

$$M^{2/3} = \frac{N[\eta]^{1/3}}{\beta} \cdot \frac{\eta_0 s_f^0}{(1 - \bar{v}\rho_0)}$$

where N is Avogadro's number, β the shape factor (2.12×10^6 for spherical particles), and η_0 is the solvent viscosity in poises. The quantity $\eta_0 s_f^0/(1-\bar{v}\rho_0) = 3.2 \times 10^{-13}$ was derived from the slope of the line in Fig. 1 by assuming $\bar{v}$ to be constant. Since spherical molecules were indicated by the viscosity (Table I) in 2.5% sodium chloride (1.014 g/ml), the computed M_w was 4.8×10^6 for the floating fraction. If the higher intrinsic viscosity in 5% sodium chloride is due to swelling or elongation, a similar weight can be computed by assuming a reasonable value for β.

Results from the Archibald (11, 12) sedimentation procedure were of limited value. Addition of heavy immiscible liquids to aid in locating the base gave spurious values and accumulation of material at the meniscus introduced uncertainties at this boundary. The mean of several measurements at both boundaries gave $M_w = 5.4 \times 10^6$ but individual observations ranged from 2 to 10×10^6. This variability is interpreted as indicating polydispersity and heterogeneity in addition to experimental error.

Lipid-free Protein

Following lipid extraction the insolubility of the protein presented the major experimental difficulty. A suitable solvent should dissolve the protein completely, have physical properties (density, viscosity, etc.) that would permit the particle size to be estimated by the usual physical methods, and cause little or no rupture of peptide bonds under the conditions of observation. No solvent was found that fully met these conditions. The lipid-free protein did not dissolve in various buffer solutions between pH 3.6 and 9.5 or in 6 M urea, and only partly dissolved in strong inorganic acids. It dissolved in formic acid, dichloroacetic acid, hydrazine, and dilute sodium hydroxide. Most of the subsequent work was done in formic acid solutions since available evidence (13, 14, 15) indicates that it is not likely to cause cleavage of the peptide bond. By adding methanol the undesirably high density and viscosity of formic acid could be reduced and this mixed solvent was used to a limited extent. Finally, since there was some evidence of aggregation in these solvents under certain conditions, a few measurements were made in sodium hydroxide solutions.

The sedimentation behavior of the lipid-free protein was determined in the selected solvents and the results are summarized in Table II. Three boundaries having different sedimentation rates were evident in these solvents (Fig. 3). These are referred to as components: A of ca. 2 S (Svedbergs), B of 4 to 8 S, and C of 16–40 S. In addition, protein concentrations above 0.3% and electrolyte concentrations above 0.05 N tended to cause gel formation in early preparations but was less evident in protein prepared by Soxhlet extraction. Solutions in which gel formation occurred were discarded.

In formic acid of 70% (w/w) concentration or higher, or in formic acid (88% w/w) diluted with less than 25% (v/v) methanol, only component A was present. Further dilution of the formic acid with either water or methanol showed the presence of both A and B components. Dilution with water

TABLE II

Sedimentation behavior of vitellenin in various solvents

Solvents and conditions*		Sedimentation rate of components present† A Sved.†	B Sved.	C Sved.
Behavior in formic acid				
1. Formic acid 88% (w/w) (This concn. designated "formic acid" below)	($s^0_{20,w}$)	2.1	–	–
2. Formic acid 71% (w/w)		2.1	–	–
3. Formic acid 64% (w/w)		1.9	4.6	–
4. Formic acid 53% (w/w)		2.3	9.2	–
Behavior in formic acid – methanol mixtures				
5. 6 to 25% methanol (v/v) in formic acid	($s^0_{20,w}$)	2.1	–	–
6. 50% methanol (v/v) in formic acid		1.6	5.7	–
7. 75% methanol (v/v) in formic acid		1.4	3.9	–
8. 100% methanol		–	6–10	–
9. 100% methanol diss. and dial. 5° C		–	7.4	–
10. 100% methanol diss. and dial. 44° C		–	–	16–40
Behavior with added electrolytes‡				
11. As 5 (25% methanol) + 0.05 *N* $NaC_2H_3O_2$		1.9	–	–
12. As 5 (25% methanol) + 0.10 *N* $NaC_2H_3O_2$		1.6	–	22
13. As 5 (25% methanol) + 0.10 *N* KCNS		1.3	–	28
Behavior in sodium hydroxide				
14. 0.1 *N* NaOH + 0.2 *N* NaCl. In soln. <1 day	($s^0_{20,w}$)	2.2	Trace	–
15. 0.1 *N* NaOH + 0.2 *N* NaCl. In soln. <1 day		2.0	Trace	–
16. 0.1 *N* NaOH + 0.2 *N* NaCl. In soln. 1 day		1.8	Trace	–
17. 0.1 *N* NaOH + 0.2 *N* NaCl. In soln. 5 days	($s^0_{20,w}$)	1.7	Trace	–
18. 0.1 *N* NaOH + 0.2 *N* NaCl. In soln. 29 days		1.5	Trace	–
Effect of varying pH				
19. As 15 adjusted to pH 10.4 with HCl		2.7	Trace	–
20. As 15 adjusted to pH 2.1 with HCl		3.0	8.7	–
21. As 20 adjusted back to pH 12.5 with NaOH		2.3	5.8	–

*Unless otherwise stated, all samples were dissolved at room temperature, and dialyzed and stored at 5° C.

†$s^0_{20,w}$ values where so indicated: all others $s_{20,w}$ at protein concentration below 1% in NaOH and below 0.5% in other solvents.

‡See Table III for NaCl additions to formic acid.

yielded a system that was extremely sensitive to traces of electrolytes, and gel formation or aggregation occurred. Protein concentrations below 0.3% in formic acid could be dialyzed against absolute methanol without precipitation. If this was done at 5° C or at room temperature, only component B was present, while preparation at 44°C gave only component C, indicating that exposure to higher temperature favors aggregation. Addition of electrolytes to solutions in methanol – formic acid gave components A and C instead of A and B.

As might be expected, the sedimentation coefficient of the protein in sodium hydroxide solution gradually decreased with time. The significant point is that initially component A has about the same sedimentation coefficient in this solvent as in formic acid. Partial neutralization or rapid acidification resulted in the formation of the B aggregate, which was otherwise evident in only trace amounts.

The variation in the sedimentation rates of components B and C suggests that they are polydisperse aggregates. As only the B component was present in absolute methanol solutions prepared at low temperature, its molecular

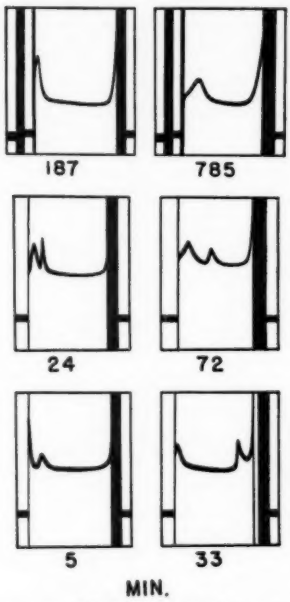

FIG. 3. Sedimentation pattern of lipid-free protein: top, in formic acid 88% w/w plus 0.05 *N* NaCl; center, in 50% v/v methanol in formic acid; bottom, in 25% v/v methanol in formic acid plus 0.1 *N* potassium thiocyanate.

weight was measured by light-scattering methods and found to be 1.2×10^6. This high value and polydispersity were also indicated by the Archibald procedure. Since this exceeds all estimates of the size of the protein moiety in any of the lipid-containing materials (1), component B must be an aggregate, and also component C, since it has a still higher sedimentation rate. Clearly, the only solvents suitable for quantitative measurement are those that can maintain the state of dispersion represented by component A.

Accurate measurements of $\bar{v}$ are necessary for molecular weight estimation in dense solvents. As inevitable evaporation losses introduce appreciable error at high salt concentration, these measurements were made in formic acid without added electrolytes. The value of 0.742 ± 0.001 ml/g so obtained was assumed to be applicable in the other solvents where the required accuracy was precluded by salt content, volatility, or corrosive action. By extrapolating the least squares plot of $\eta_0 s^0$ vs. ρ_0 (Fig. 4 with η_0 in centipoises) in the range of formic acid – methanol mixtures that yielded only the A component, a similar $\bar{v}$ was obtained, although this was subject to the relatively large error of the intercept.

The results of the physical measurements on the lipid-free protein are summarized in Table III. By extracting the organic solvents, used to remove the lipid, at low temperature, it was possible to obtain preparations that would tolerate 0.30 *N* sodium chloride in the formic acid solvent without evidence of aggregation. It was found experimentally that 0.20 *N* sodium chloride was

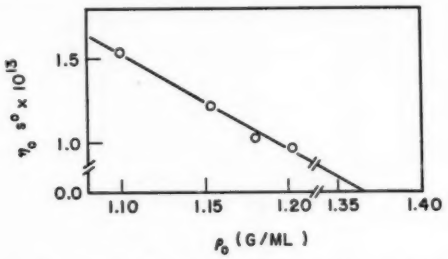

FIG. 4. Sedimentation rate ($\eta_0 s^0$) as a function of solvent (methanol – formic acid) density.

TABLE III

Summary of measurements of vitellenin

Property		Formic acid* +0.2 *N* NaCl	Formic acid +0.05 *N* NaCl	0.1 *N* NaOH +0.2 *N* NaCl
$dn/dc \times 10^3$ at λ 5780	dl/g	—	1.52	1.98
$dn/dc \times 10^3$ at λ 4360	dl/g	—	1.57	—
$\bar{v}_{20}$†	ml/g	0.742	0.742	0.742
$[\eta]_{20}$	dl/g	—	—	0.13‡
$D^0_{20,w} \times 10^7$ (or conc. given)	cm^2/sec	2.86	3.43 (0.4%)	—
$s^0_{20,w}$	Sved.	2.80	2.46	2.2, 1.7‡
$M_w \times 10^{-4}$				
From s/D above		9.3	6.9	—
From $[\eta].s$		—	—	2.9‡
From Archibald procedure				
$M_n \times 10^{-4}$	Initial	8.3	—	—
	Final	3.9	—	—
From osmotic at 7° C		—	3.5†	8.5±3.6

*Designates formic acid 88% (w/w).
†Reported value measured in formic acid without added salt (see text).
‡Denotes values in solutions 5 days old.

adequate to suppress charge effects. Diffusion coefficients measured by schlieren methods in formic acid – 0.20 *N* sodium chloride showed no concentration dependence. In formic acid – 0.05 *N* sodium chloride the value reported was obtained by the integral fringe method at a 0.4% protein concentration.

The molecular weight computed from the sedimentation and diffusion coefficients in formic acid – 0.20 *N* sodium chloride is considered the most reliable and gave $M_w = 9.3 \times 10^4$. When this solvent and the Archibald procedure were used, the initial readings yielded comparable values that fell to 3.9×10^4 at the termination of the run, indicating polydispersity. The above measurements were also made in 25% methanol – formic acid, but electrolytes had to be omitted since they caused aggregation (Table II). These results are not reported in Table III since they were low ($M_w = 2.3 \times 10^4$ or less) and are believed to be subject to charge effects. Polydispersity was again indicated by the Archibald procedure. The low value obtained from $[\eta]$ and $s^0_{20,w}$ values in sodium hydroxide solution is doubtless due to molecular cleavage, although size estimates involving intrinsic viscosity are also subject to other limitations.

Estimates of the number average molecular weight (M_n) were attempted by osmotic pressure methods. These measurements were supplemented by dry

weight measurements on solution and diffusate to determine whether peptides or other dialyzable material appeared during the measurements. None was detected. The results of the osmotic pressure measurements were far from satisfactory. In formic acid some series showed a negative π/cRT vs. c plot suggestive of aggregation, although electrolytes were omitted. This behavior and the general variability precluded objective extrapolation, and the M_n ca. 3.5×10^4 reported in Table III is the average of some 14 measurements at concentrations below 0.5%.

Solutions for osmotic pressure measurements in sodium hydroxide were prepared at room temperature, centrifuged, and placed in the osmometers without dialyzing. Eight hours later the pressure head was adjusted to the expected range and the initial readings taken. The osmotic pressure decreased slightly, reached a rather flat minimum for 36 hours, and then increased slowly but continuously over a 5-day period. These values were plotted against $1/t$ and tangents to this curve after 44 and 120 hours of exposure to alkali extrapolated to infinite time. If equilibrium had been attained within the 44-hour period, M_n was ca. 8.5×10^4, but after 120 hours, when obvious breakdown had occurred, M_n was ca. 1.5×10^4. Any value between these extremes could be obtained by drawing a tangent at an intermediate time.

Discussion

Present results must be considered with those reported earlier for ether-extracted lipovitellenin (1). Since the floating fraction and lipovitellenin have partial specific volumes of 1.02 and 0.88 ml/g, respectively, they would have separated on centrifugation had the latter existed as a separate entity. Lipovitellenin must therefore interact with sufficient molecular or micellar lipid to reduce the density below that of the solvent. Indeed, there is a possibility of equilibrium between the several possible forms that could exist. Whatever the structure, ether extraction removes lipid that is essential for the maintenance of the original solubility and stability.

No evidence was obtained to indicate the presence of protein-free lipid micelles. Since $\bar{v}$ of the floating fraction was 1.02 ml/g and assuming that this decreases (1) 0.003 ml/g/% lipid decrement, removal of more than 10% lipid would result in its sedimentation in solvents of $\rho = 0.01$. Such sedimentation did not occur and as centrifugal fractionation was unsuccessful, it follows that less than 10% of the lipid occurred as "free" micelles and any that did exist must have flotation properties similar to the protein-containing particles. If the lipid removed by ether extraction (80%) was associated with the number of kinetic units present in the extracted lipoprotein, the particle size of the unextracted material should be five times as large. The presence of independent protein-free micelles would reduce M_w by increasing the number of kinetic units and their v would have favored separation had their size equalled or exceeded the mean size of the complex. While one recognizes the experimental limitations, the observed particle size of the floating material equalled or exceeded this calculated value, indicating that the amount of protein-free micellar lipid must be small.

Although the high (lipovitellin) and low density (lipovitellenin) lipoproteins of egg yolk can be separated by centrifuging, it is only after ether extraction that the latter has the lipid content (ca. 40%) of lipovitellenin (2). Sugano (16) has termed the major electrophoretic component in each of these fractions α- and β-lipovitellin and has suggested that a minor γ-component may also be a lipoprotein. The loss of solubility and ease of fractionation following ether extraction suggest that lipovitellenin is an intermediate product derived from this treatment. Since the characterization of lipovitellenin on the basis of solubility or lipid content may have little significance, attention was turned to the protein moiety, vitellenin.

If the protein moiety of the floating fraction and the ether-extracted lipoprotein (1) contain only one protein molecule, its approximate size can be estimated from the molecular weight and lipid content of these fractions. This computation gives values ranging from 1.6 to 4.8×10^5. Since this variation arises from heterogeneity and experimental errors, the observed molecular weight of the lipid-free protein would have to be less than the minimum calculated value to be considered significant. The most homogeneous fraction of this lipoprotein gave, on computation, M_w and $M_n = 1.6\times10^5$, for the protein moiety, making this figure the most acceptable experimentally.

In the present study the most reliable estimate of size gave $M_w = 9.3\times10^4$ for the lipid-free protein or about half the minimum estimate of the size of protein moiety in the lipid-containing materials. The number of polypeptide chains in the original moiety cannot be stated exactly but, as the above figure was also the highest, all results support the conclusion that two *or more* polypeptide chains are present in each particle of floating fraction or ether-extracted lipovitellenin. The presence of more than two N-terminal amino acids (17) in this protein also suggests that several polypeptide chains are present.

There was qualitative evidence of polydispersity of the protein fraction, as indicated by peak-spreading, a decrease in molecular weight with time by the Archibald procedure, and an M_n that was lower than the M_w in formic acid. Even two polypeptide chains differing in size or composition could therefore yield two, or even three "lipovitellenins," if all combinations are possible. Since secondary rather than primary valence appears to be responsible for the combination of these polypeptide chains, a still greater range of polydispersity could occur in the natural product. Other than the shoulder on the leading edge of the flotation pattern (Fig. 2), there was no evidence of discrete components, but the high lipid content may preclude their separation. However, polydispersity of the protein fraction, in addition to a variable lipid content (1), doubtless contributes to the observed heterogeneity of these lipid–protein complexes.

These results pose the question as to whether the native protein moiety is made up of several polypeptide chains held together by bonds vulnerable to the solvent used, or separate protein molecules held together by lipid. Since several enzymes have been dissolved in formic acid and recovered with unimpaired activity and without apparent changes in optical activity (14, 18, 19), it would appear that neither peptide nor disulphide bonds are likely to be ruptured. Therefore, the structure of the floating fraction and ether-extracted

lipovitellenin must resemble that of a micelle, since both the lipid and protein portions are made up of smaller units that combine through secondary valence.

Acknowledgments

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FURTHER STUDIES ON THE METABOLISM OF GLUCOSE AND THE FORMATION OF CORTICOSTEROIDS IN VITRO[1]

E. Schönbaum, Monica Davidson,
Rosemary E. Large, and W. G. Bruce Casselman

Abstract

Glucose, adenosine, uridine, and reduced triphosphopyridine-nucleotide (TPNH) significantly increase the effect of corticotrophin (ACTH) on the formation of steroids by rat adrenals in vitro. This effect of glucose is inhibited by 2-deoxyglucose. Inhibition is overcome by TPNH. During the incubation of adrenal fragments, but not of intact glands, TPN-dependent dehydrogenases leak into the medium.

Introduction

Earlier studies have shown that the stimulation by ACTH of steroid formation by rat adrenals in vitro is enhanced by glucose, unaffected by added insulin or adrenaline, and virtually prevented by anaerobic conditions (1). The experiments described in this paper concern carbohydrate metabolism and steroid synthesis in adrenal tissue. In agreement with the conclusions of Haynes (2), Koritz (3), and Pincus (4), the results indicate that the reduction of TPN plays an important role in the immediate response of the adrenal cortex to ACTH. As in previous studies (5) there was no relationship between the rate of steroid formation and the concentration of ascorbic acid either in the gland or added to the medium.

Methods

Male and female rats of the Wistar strain (Woodlyn Farms, Guelph, Ontario) (150–250 g) were used in all experiments. The adrenals of several rats were prepared and incubated as described previously (1, 5), except that the tissue was usually preincubated in glucose-free Krebs–Ringer bicarbonate medium. In each experiment the adrenal quarters were divided among four or eight flasks so that each flask contained one quarter from each animal. This arrangement allows the application of the paired t test to the differences between rates of steroid formation in the various flasks. Substrates, inhibitors, and corticotrophin (ACTH, 200 milliunits/100 mg tissue) were added at the beginning of the incubation period. Steroids were estimated by their absorption at 240 mμ in CH_2Cl_2. For the sake of convenience, the results were expressed in terms of the amount of hydrocortisone which would give the observed optical densities (6). Glucose was estimated by the method of Somogyi and Nelson (7).

Results

The results of studies on steroid formation and glucose utilization by adrenal tissue in vitro are presented in Table I. While ACTH significantly increases

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Contribution from the Banting and Best Department of Medical Research and the Department of Anatomy, University of Toronto, Toronto, Ontario. Parts of this work have been presented at scientific meetings.

TABLE I

Steroid formation and glucose utilization

	Steroid formation				Glucose utilization		
Expt.	ACTH*	Glucose (No ACTH)	Glucose + insulin	Glucose + ACTH	Glucose† (No ACTH)	Glucose + insulin	Glucose + ACTH
A‡	100	75	66	150	100	71	97
B	100	86	86	133	100	177	120
C	100	58	58	100	100	236	57
D	100	36	50	131	100	552	110
E	100	77	77	162	100	135	114
F	100	50	55	123	100	175	122
G	100	42	38	168	100	141	11
Mean		61	61	138		212	90

*Mean steroid production in seven experiments using quartered adrenals stimulated by ACTH was 18.4 ± 1.99 μg (as hydrocortisone) per 100 mg tissue per hour. The figures indicate per cent of the control containing ACTH only. ACTH: 0.2 I.U. per 100 mg tissue. Insulin: 10 I.U. per ml medium.

†The utilization of glucose in the absence of insulin or ACTH was 193.4 ± 26.4 μg/100 mg tissue per hour. The figures indicate per cent of the control containing glucose only.

‡Each letter represents an experiment with tissue taken from the same rats.

the rate of steroid formation it does not appreciably affect the uptake of glucose. On the other hand, insulin, while not increasing the output of steroids, stimulates the uptake of glucose, though this effect is rather variable. In another series of experiments it was observed that insulin has no effect on the formation of steroids in the presence of both ACTH and glucose. Without insulin the rate of formation of methylene chloride soluble, U.V.-absorbing material was 27.2 μg/100 mg/hour (as hydrocortisone) and with insulin, 27.8, while the mean of the differences (paired t test) was 0.5 ± 2.5 ($n = 8$).

Table II summarizes the results of comparative studies of the effects of various substances upon the rate of formation of steroids in the presence of ACTH. The control substrate, if any, and the rate are given in columns 1 and 2, and the experimental substrate and rate in columns 3 and 4. Δ in column 5 represents the mean $\pm$ standard error of the differences for the individual experiments. The number of experiments (n) and the significance of the differences are given in columns 6 and 7. The results were analyzed by the paired t test because each flask contained similar amounts of tissue from each animal (see Methods).

Screening experiments showed that mevalonic acid, amino acids, and several Krebs cycle acids had no effects comparable to that of glucose. These substrates are not included in Table II. Like glucose, adenosine and uridine (see Table II) do enhance the effect of ACTH. In the present studies adenine and ribose had no statistically significant effect although previously (1) there was slight enhancement with ribose.

Some experiments with 2-deoxyglucose (2-DG) were intended to study the mechanisms involved in the effects of insulin and ACTH. 2-DG ultimately acts as an inhibitor of phosphohexose isomerase (8). For inhibition to occur, the fragments of rat adrenal tissue must be preincubated with the inhibitor. The results indicate that such treatment largely blocks the increase in the rate

TABLE II

Effects of various substrates on ACTH-stimulated steroid formation

Control			Test					
Substrate I		U.V.^{I}*	Substrate II		U.V.^{II}	△†	n	P
None		23.1	Glucose	0.01 *M*	32.0	9.5±1.5	18	<0.01
None		23.5	Adenosine	0.01 *M*	40.0	16.6±2.6	6	<0.01
None		25.8	Uridine	0.01 *M*	32.0	6.0±2.2	6	<0.05
None		23.0	Adenine	0.01 *M*	23.0	− 0.2±3.3	4	>0.1
None		23.0	Ribose	0.01 *M*	27.0	4.2±2.1	4	>0.05
None		23.7	Glucose	0.01 *M*	32.7	9.0±1.9	6	<0.01
None		37.5	Adenosine	0.01 *M*	51.2	13.7±3.9	6	<0.02
None		32.3	2-DG	0.01 *M*	29.9	0.6±0.8	14	>0.05
Glucose	0.01 *M*	32.7	Glucose + 2-DG	0.01 *M*	25.3	− 7.3±2.3	6	<0.05
Adenosine	0.01 *M*	48.4	Adenosine + 2-DG	0.01 *M*	38.0	−10.4±3.8	5	>0.05 <0.1
None		24.0	Glucose	0.01 *M*	30.0	6.0±1.4	22	<0.05
None		26.3	G-6-P		25.5	− 1.0±1.4	16	>0.05
Glucose	0.01 *M*	32.4	G-6-P	0.01 *M*	25.1	− 7.2±0.9	16	<0.02
None		24.3	TPN	0.001 *M*	24.9	0.6±1.8	10	>0.1
None		19.9	TPNH	0.001 *M*	26.0	6.1±1.3	8	<0.005
Glucose	0.01 *M*	35.9	Glucose + TPN		39.0	3.1±3.0	8	>0.05
G-6-P	0.01 *M*	32.9	G-6-P + TPN		59.8	26.9±3.7	16	<0.002
Adenosine	0.01 *M*	36.0	Adenosine + TPN		35.7	− 0.3±2.6	6	>0.1
G-6-P	0.01 *M*	43.7	G-6-P + 2-DG		34.3	− 9.6±2.2	6	<0.01
G-6-P + 2-DG	0.01 *M*	34.3	G-6-P + 2-DG + TPN		57.5	23.1±5.3	6	<0.01

*The rate of formation of steroids (U.V.^{I} and U.V.^{II}), as well as △, is expressed in μg (as hydrocortisone) per 100 mg adrenal tissue per hour.

†△: This figure does not represent the difference between the mean values; it represents the mean ± S.E. of the differences found in the individual experiments.

P: Refers to the paired *t* test.

of formation of steroids due to the addition of glucose, glucose-6-phosphate, or adenosine.

Another series of experiments concerned the use of glucose-6-phosphate (G-6-P) as substrate. It is not as effective as glucose. However, in the presence of triphosphopyridine-nucleotide (TPN, the oxidized form), G-6-P tremendously enhances the formation of steroids, while no such effect is obtained with glucose plus TPN or TPN only. TPNH enhances the formation of steroids significantly. These results are somewhat similar to those obtained by Koritz and Péron (3), who added both glucose and G-6-P, together with TPN. To explore the mechanisms of these effects, adrenal quarters were preincubated in Krebs–Ringer bicarbonate medium, and subsequently the medium only was tested for the presence of G-6-P and 6-phosphogluconate dehydrogenase. G-6-P was added to the medium (0.01 *M* final concentration) and changes in transmittance at 340 mμ recorded with a Beckman-DU spectrophotometer and a Varian recorder. The rate of decrease in transmittance following the addition of 0.001 *M* TPN (final concentration) corresponded to that for a zero-order reaction (Fig. 1). In a similar experiment with carefully dissected, whole adrenals there was no change in absorption at 340 mμ. These observations show that TPN-dependent enzymes using G-6-P as substrate, leak into the medium from the cut adrenals during preincubation as well as during incubation. No leakage of hexokinase could be demonstrated using glucose, TPN, and adenosine-triphosphate.

A series of experiments concerning the effects of ascorbic acid upon the rate of formation of steroids showed that the addition of 0.01 *M* ascorbic acid did not affect the rate of formation of steroids in vitro, either in the presence or absence of ACTH. Likewise, if adrenal tissue depleted of ascorbic acid (adrenal

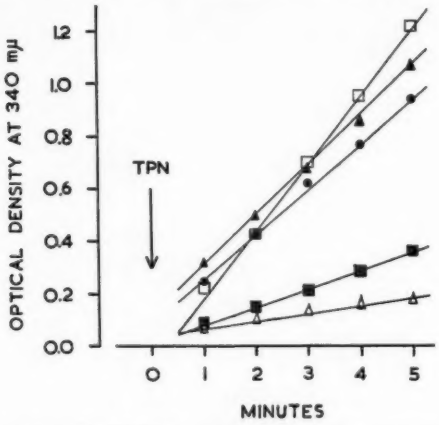

FIG. 1. The formation of TPNH by enzymes leaking from quartered rat adrenals incubating at 38° C into Krebs–Ringer bicarbonate medium.

1. Adrenal tissue was incubated for 90 minutes; the medium was changed every 15 minutes. ●, ▲, △: dehydrogenase activities found in the media after the 1st, 2nd, and 6th 15-minute period.

2. Adrenal tissue was preincubated for 30 minutes and then incubated for 1 hour. □, ■: dehydrogenase activities found in the preincubation and incubation media respectively.

TPNH was measured at 340 mμ in quartz cuvettes of 1-cm path length containing 0.4 ml medium to which G-6-P and TPN (10 m*M* and 1 m*M* final concentrations) were added.

tissue taken from rats injected with histamine (5)) was incubated in a medium containing 0.12 *M* sodium ascorbate instead of sodium chloride, there was no significant difference in the output of steroids (29 ± 3.9 and 30 ± 3.3 μg/100 mg/hour of methylene chloride soluble, U.V.-absorbing material (as hydrocortisone) respectively).

Discussion

All data, 246 in total, were subjected to an analysis of variance. In so doing, we sacrificed the principle of equal distribution of any one pair of adrenals over all flasks in view of the unequal number of observations in any one treatment. The analysis reveals a highly significant substrate effect, but it obscures completely some of the highly significant differences indicated by the paired *t* test. This demonstrates the importance of the proper experimental design, namely, the equal distribution of tissue from any one pair of adrenals over all flasks.

The results presented in Table I show that the response of the adrenal cortex to ACTH in vitro is greater (see also (1)) in the presence of glucose and suggest that the availability of glucose might be a rate-limiting factor, while on the other hand an increased disappearance of glucose in the presence of insulin does not seem to be sufficient to increase the rate of formation of steroids. The results of the substrate studies (Table II) indicate that not only in cell-free adrenal preparations (9) but also in tissue slices, the pentose cycle is of considerable importance. Thus, adenosine and uridine significantly increase the

formation of steroids under the stimulus of ACTH. A similar effect has been observed for inosine by Dr. Marion K. Birmingham, of McGill University, Montreal, Quebec (personal communication). Presumably, these substances increase the metabolism of the ribose phosphates and, therefore, the reduction of TPN. The availability of TPNH appears to be one of the important determinants of the rate of steroid formation. This is also borne out by the results shown in Table II. TPNH but not TPN (in the absence of G-6-P) increases the formation of steroids. 2-DG could inhibit steroid formation by decreasing the recycling of ribose phosphates through its inhibition of phosphohexose isomerase and thus reducing the supply of TPNH. G-6-P is probably not converted to glucose because there is no glucose-6-phosphatase in the adrenals (personal communication by Dr. R. Hawkins, Department of Physiology, University of Toronto). However, possibly G-6-P-dehydrogenase leaks into the medium and acts on G-6-P with reduction of TPN so that the increased production of steroids, even if 2-DG is present, might be due to the TPNH. Alternatively, the effect of G-6-P might lead to the unlikely suggestion that the phosphate might enter the cells of the adrenal cortex more readily than glucose. However, we have been able to demonstrate the presence of G-6-P-dehydrogenase in media after incubating quartered adrenals. Thus it would appear that our results with G-6-P and TPN as well as those of Koritz and Péron (3) can be attributed to reduction of TPN in the medium. In the same way added TPN might be reduced to TPNH to overcome the inhibition by 2-DG (Table II). That G-6-P + TPN is more effective than TPNH might be attributed to the continual supply of TPNH in the first case in contrast to the limited, initial supply in the second.

Most results presented in Tables I and II, as well as some of those published previously (1), are in agreement with present concepts of the action of ACTH, namely the activation of adrenal phosphorylase, first reported by Haynes and Berthet (2). Increased glycogenolysis would result in an increased availability of TPNH. In that case, glucose or adenosine should increase the output of steroids in the absence of ACTH. This is not borne out by the experimental data. Therefore one cannot explain the effects of glucose or adenosine on the basis of activation of phosphorylase by ACTH, unless one would postulate the formation of glycogen as an obligatory step in the mechanism of action of these substrates.

The leakage of the TPN-linked dehydrogenases might account for the lower rate of formation of steroids by adrenal slices in vitro as compared to glands in vivo or perfused. While the studies with tissue slices retain their value for comparisons, this finding indicates one more problem in the direct application of results obtained with tissue slices to conditions existing in the intact animal.

Ascorbic acid is another substance that leaks into the medium even when whole adrenals are incubated (10). However, the absence of any effect of added ascorbic acid on adrenal tissue from normal or histamine-treated rats suggests that such a loss of the acid will not affect the rate of steroid formation. Thus the relationship between adrenal ascorbic acid levels and steroidogenesis remains unsolved.

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Addendum

In a very recent communication, R. C. Haynes, Jr., S. B. Koritz, and F. G. Péron (J. Biol. Chem. **234**, 1421, June 1959) reported that adenosine 3′,5′-monophosphate enhanced the formation of steroids by rat adrenals incubating in Krebs–Ringer–phosphate buffer containing 200 mg% of glucose. This response was obtained in the presence as well as in the absence of ACTH. 2′-, 3′-, or 5′-Adenylic acid were without effect. This suggests that the effects of adenosine described in this paper were probably not due to an activation of phosphorylase (Haynes, R. C., Jr. J. Biol. Chem. **233**, 1220 (1958)). Whether adenosine 3′,5′-monophosphate would enhance the formation of steroids in the *absence* of glucose cannot be said at present.

A SIMPLE METHOD FOR THE ASSAY OF SMALL AMOUNTS OF ANTIDIURETIC HORMONE[1]

J. HUNTER, H. KALANT, AND J. C. OGILVIE

Abstract

A reliable, sensitive, and simple technique is described for the assay of antidiuretic hormone in amounts between 25 and 130 μunits. The assay animal is a conscious albino rat weighing between 150 and 250 g. On the day of assay it is tranquillized with an injection of perphenazine. Specially designed cannulae, implanted on the day before the assay, provide unimpaired access to the stomach and bladder, permitting the maintenance of hydration and continuous urine collection without disturbance of the animal. Material to be assayed is injected intravenously. Water load is maintained by manual injection of 0.5-ml quantities of 0.05% NaCl solution at intervals dependent upon the rate of urine production. A useful index for expression of the response is described, which is related to both the intensity and the duration of antidiuresis. Assay of an artificial unknown by this method gave a result within 6% of the true value, and an excellent statistical precision ($\lambda = 0.143$).

Introduction

The posterior pituitary antidiuretic hormone (ADH) is an extremely potent material which produces its characteristic effects on renal tubular function at concentrations much too low to be measured by chemical means. For this reason, sensitive and reliable estimation of ADH activity in body fluids and tissues is dependent upon bio-assay. Numerous methods have been described (cf. recent review by Thorn (2) and Kaufmann (3)), of which the most sensitive are variants of the procedure first described by Dicker (4). This consists of the electronic measurement of urine flow rate, before and after intravenous administration of the ADH or unknown material, in rats anaesthetized with alcohol and maintained in a constant state of hydration by automatic replacement of the water load at the same rate as it is lost in the urine. Experience in this laboratory and elsewhere (5, 6) has shown that the Dicker method often fails because of the critical adjustment of narcotization required. Too much alcohol results in complete suppression of urine flow, due to circulatory collapse. Insufficient alcohol results in urine suppression possibly due to neurogenic stimuli in the incompletely sedated animal.

Ginsburg and Heller (7) attempted to overcome this difficulty by the use of a fully conscious test animal, in which the cannula for intravenous administration of ADH had been inserted on the day before assay. These authors, however, hydrated the rats by repeated gavage, and obtained urine by digital pressure on the bladder. Such repeated handling incurs the risk of endogenous ADH release, which may account for the variability in sensitivity found by Ginsburg and Heller.

Kellogg, Burack, and Isselbacher (8) described an ingenious method of maintaining a continuous water load in fully conscious rats for periods of several

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days, via an indwelling gastric catheter. However, in ADH assays involving accurate measurement of urine output over short intervals, it has been our experience that the presence of a catheter near the larynx can induce reflex inhibition of urine flow.

We have found that these difficulties can be overcome by using a conscious animal, previously treated with perphenazine (Trilafon (R), Schering Corp.), which minimizes urinary inhibition resulting from extraneous stimuli. Such an animal, hydrated via a gastric fistula, maintains a satisfactorily constant rate of urine flow. In addition, the measurement of urine flow and the maintenance of water load can be effectively performed by an extremely simple apparatus, which is described below.

Methods

Male albino rats weighing from 150 to 250 g are used. On the day preceding the assay, the animals are anaesthetized with 12% ethanol by gavage, supplemented when necessary by ether, and specially made indwelling cannulae are inserted into the stomach and the urinary bladder through mid-line abdominal incisions. These cannulae are made of brass, and have the shape and dimensions shown in Fig. 1. The bladder cannula should be implanted carefully, with its longitudinal axis coinciding with that of the bladder, in order to prevent occlusion of the drainage hole. The bladder wall is tied securely to the shaft of the cannula, and bladder tissue peripheral to the ligature is excised, reducing the bladder volume to a minimum. The shafts of the cannulae are anchored firmly to the muscle and skin of the abdominal wall, with the collars resting on the skin and the threaded ends protruding, so that they can be easily covered with threaded brass caps (Fig. 2).

On the day of assay the caps are removed and polyethylene tubing is attached to the ends of the brass cannulae by means of threaded adapters. This arrangement provides direct entry into the stomach for purposes of hydration, and unimpeded urinary flow for volumetric measurement. Initially, a polyethylene cannula was introduced into the external jugular vein on the day preceding the assay, but some assays were not successful because of occlusion or dislodgement of the cannula, and therefore this step is now carried out on the day of assay. The animals are given 1.0 mg of perphenazine per 100 g of body weight by intraperitoneal injection, after which the jugular cannula, made of polyethylene tubing of 0.58 mm I.D., 0.965 mm O.D. (PE-50, Clay-Adams Co.), is inserted under the lightest possible ether anaesthesia. Initial hydration, with a fluid load amounting to 7–8% of body weight, is carried out by syringe via the gastric cannula. Additional hydrations by the same route are carried out during the subsequent assay, 0.5 ml of fluid being injected for each 0.5 ml of urine produced. It has not been found necessary or desirable to make any allowance for extrarenal loss of water.

Hydration was originally carried out with tepid tap water. In later work, a 0.05% NaCl solution has been used. This change was made on the basis of a number of determinations of total electrolyte loss during the course of an assay procedure. Despite Thorn's statement (2) that there is no rationale for

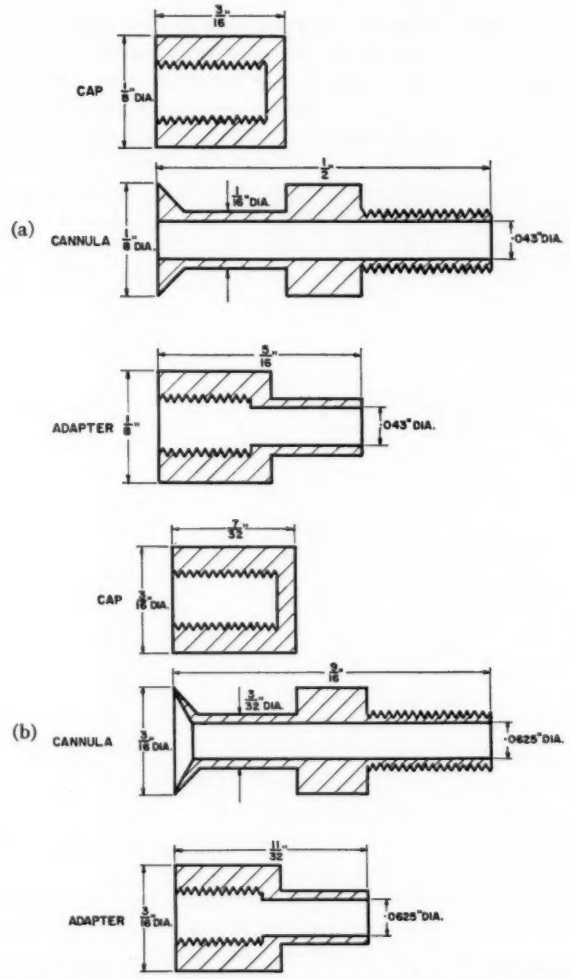

FIG. 1. Size and dimensions of (*a*) stomach cannula and (*b*) bladder cannula.

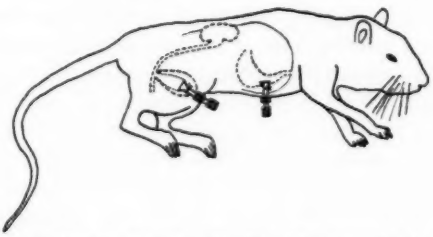

FIG. 2. Stomach and bladder cannulae *in situ*. The cannulae have threaded caps in place.

the replacement of electrolyte, our observations to date suggest that this salt solution is more effective than water alone in maintaining a constant rate of urine flow throughout an assay period of 8 hours or more. Paulisch (5) and Sawyer (9) employ a similar dilute saline solution with excellent results.

The rat is placed in a small wooden box with a slot in the bottom to permit passage of the gastric and vesical cannulae. Provided direct draughts are avoided, this box helps to maintain a reasonably constant immediate environment. The free end of the vesical cannula is inserted into the top of a microburette with graduations of 0.05 ml. The completed preparation is shown in Fig. 3. When a constant urine flow of at least 0.1 ml per minute has been at-

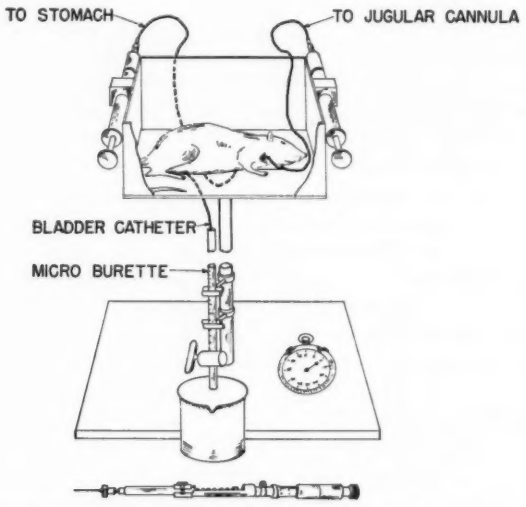

FIG. 3. Completed assembly for ADH assay. (*a*) Schematic drawing. (*b*) Photograph.

tained the output is measured in successive 1-minute intervals. Visual recording of urine volume in this manner is tedious, but is much simpler and more reliable than elaborate electronic drop counting. An initial injection of 0.3 ml of freshly prepared physiological saline solution into the jugular cannula serves as a check on the patency of the cannula, and on the normal behavior response of the rat. This injection should never cause antidiuresis, the normal response being either no change in urine flow, or a slight transient increase. Pitressin $^{(R)}$ (Parke, Davis & Co.), dissolved in distilled water acidified with two drops of glacial acetic acid per 100 ml, is delivered from a micrometer syringe ("Agla", Burroughs-Wellcome Co.) into the jugular cannula, in amounts of from 25–140 μunits (preferably in a constant volume not exceeding 0.1 ml), and flushed in with 0.2 ml of saline. The urine flow is allowed to return to a stable level before further doses of Pitressin $^{(R)}$ are given. Up to nine injections have been given to the same rat in the course of 1 day, with no apparent loss of responsiveness. This is in agreement with the observations of Dettelbach (6), who reported that

PLATE I

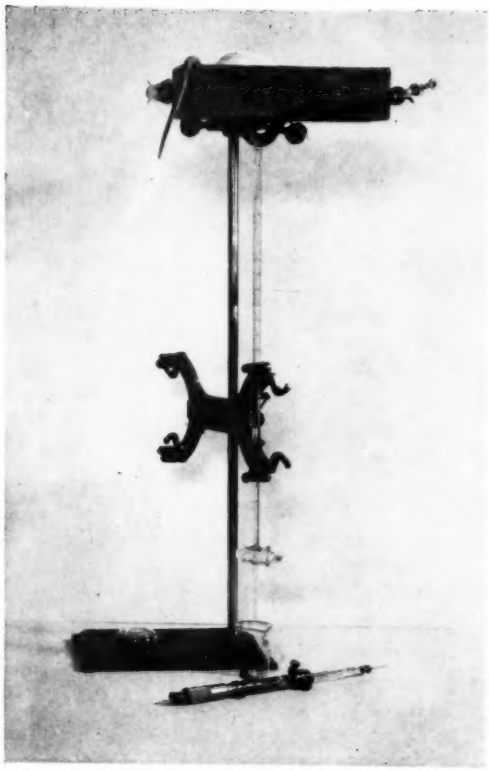

Fig. 3*b*

he could find no evidence of tachyphylaxis. The first injection was often observed to give variable responses. The practice was therefore adopted of giving a priming dose of 70 μunits, once an adequate diuresis was established, the response not being included in the evaluation of subsequent doses.

The average rate of urine flow is determined for the 5-minute period preceding the injection, and the 5-minute period immediately after recovery from inhibition. The period of inhibition is taken as the time immediately following injection until the urine flow rate regains a stable value after recovery from inhibition. The actual output is the measured volume of urine put out during this period of inhibition. The calculated output is the amount which would have been put out had the urine flow continued throughout at a rate represented by the mean of the pre- and post-injection flow rates. The difference between the calculated and actual outputs is referred to as the "inhibited volume". The dose–response curve is obtained by plotting log dose against log inhibited volume.

Results

Unoperated rats injected with perphenazine in the dosage used here, and hydrated to the same extent by stomach tube, show a fully normal diuretic response. Control rats subjected to the complete operative procedure described above including injection of perphenazine, and maintenance of water load via the gastric cannula, but not used for ADH assay, showed a delay of 1.5 to 2 hours in reaching a rate of urine flow suitable for assay purposes. This delay is attributable to the ether anaesthesia used during cannulation of the jugular vein. During the succeeding 4 to 6 hours the rate of urine output rose gradually and steadily to final levels which were from 25–100% above those at the start of this period. This rise was so gradual that the assumption of a uniform base line (the mean of the pre- and post-injection levels) during the assay period of a single dose of ADH is justifiable. However, for maximum accuracy in assays, comparable doses of standard and unknown should be tested in sequence, rather than separated by a prolonged interval of time.

In Fig. 4 are illustrated the results of a typical experiment with standard Pitressin solutions, representative of more than 50 such trials. Three doses of Pitressin (25, 50, and 100 μunits) were given, and repeated. A gradation of response is evident, and there is evidence of a fair degree of reproducibility of the response at each dose level. The data for this particular experiment are tabulated in Table I. The plot of log dose against log "inhibited volume" for this rat gives the relationship illustrated in Fig. 5.

Once experience in the technique is acquired, it is possible to obtain such results on most of the rats set up, and there is therefore no necessity for preselection of the animals.

For statistical evaluation of the reproducibility of response to standard Pitressin solutions, only those trials were used in which at least two injections had been given at each of three dose levels. In the first group of 15 rats three dosage levels of Pitressin were used: 25, 50, and 100 μunits. Each animal was tested at all three levels which were then repeated in a different order. The

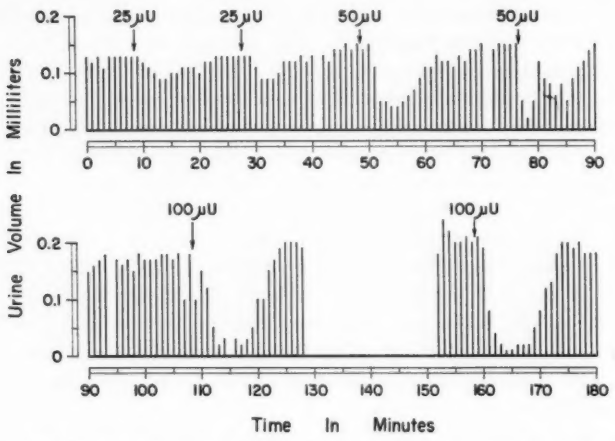

FIG. 4. Antidiuretic responses to Pitressin injected intravenously into 220-g rat. For calculation of responses, see Table I.

TABLE I

Data and calculations of typical standard response (cf. Fig. 4)

Dose, μU	Actual urine output (ml)	Calculated urine output (ml)	Inhibited volume (ml)
25	1.64	1.95	0.31
25	1.23	1.43	0.20
50	1.94	2.80	0.86
50	1.21	2.10	0.89
100	1.12	2.70	1.58
100	1.38	3.20	1.82

response used was log inhibited volume. This response measure satisfied the two statistical criteria given by Bliss (10). Log inhibited volume was found to be a linear function of log dose and the variances at the three dose levels were homogeneous. The dose–response line for the two tests is shown in Fig. 6. The analysis of variance is given in Table II. The slope of the line was 1.62 and λ was equal to 0.173. There were significant differences in average response between animals. Also there was a significant difference between tests, that is between the first and second set of three dose levels. This difference meant that response sensitivity had changed between the first and second tests. The possibility of using preinjection flow rate as a covariate to reduce variation due to changes in response sensitivity was examined but this approach did not prove to be profitable. However, the precision of the comparison of the standard with an unknown may be improved by keeping doses at the same level close together in time.

In the second group of nine rats there were as before dosage levels of 25, 50, and 100 μunits of Pitressin but with the two tests at each level being given

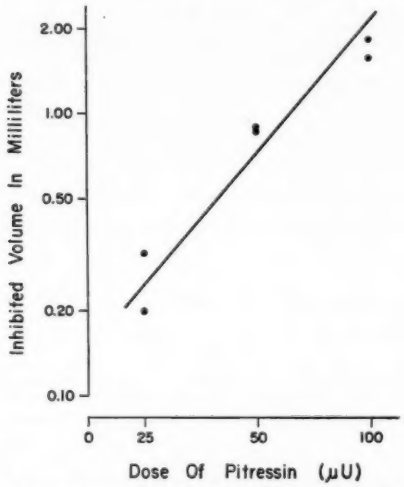

FIG. 5. Log dose – log response plot of results from experiment illustrated in Fig. 4.

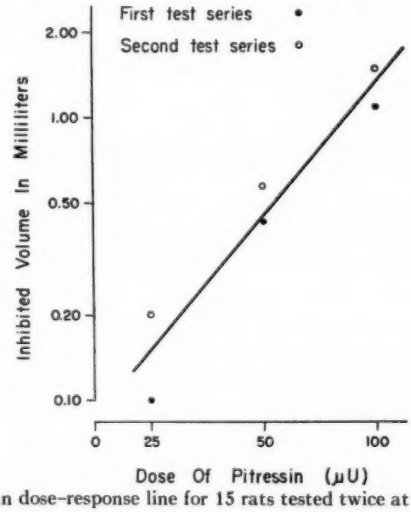

FIG. 6. Mean dose–response line for 15 rats tested twice at each dose level.

consecutively. The analysis of variance is given in Table III. This design produced a satisfactory reduction of error variation but there was significant curvature. The 25-μunit doses were the first to be given to most of the animals, which suggested that the depressed response to this dose level was due to a lower sensitivity of the animals at the beginning of the dosage series. There was the additional possibility that the 25-μunit dose was not quite on the linear portion of the dose–response curve.

Both these factors were taken into account in the last group of four rats which were used for a 3×2 assay with an artificial unknown. An initial test

TABLE II

Analysis of variance of responses of 15 rats to three dose levels of Pitressin given twice

Source	D.F.	Mean square
Rats	14	0.3703*
Tests	1	0.9242*
Doses		
D_1, linearity	1	14.2496*
D_2, curvature	1	0.0794
TD_1	1	0.3375
TD_2	1	0.0720
Remainder	70	0.0788

*Significant at the 1% level.

TABLE III

Analysis of variance of responses of nine rats to paired doses of Pitressin at three levels

Source	D.F.	Mean square
Rats	8	0.2105*
Doses		
D_1, linearity	1	9.6203*
D_2, curvature	1	0.2997*
RD	16	0.0495
Within pairs of doses	27	0.0305

*Significant at the 1% level.

dose of 70 μunits of Pitressin was given, the response to it being ignored in the calculations. The dose levels for the Pitressin standard and the unknown were 35, 70, and 140 μunits. The order of the three dose levels in each animal was random with doses at the same level of standard and unknown being given consecutively in random order. The analysis of variance is given in Table IV.

TABLE IV

Analysis of variance of a 3×2 assay of Pitressin and an artificial unknown based on four rats

Source	D.F.	Mean square
Rats	3	0.9575*
S vs. U	1	0.0280
Linearity	1	3.6672*
Curvature	1	0.1452
Departures from parallelism	1	0.0110
Opposed curvature	1	0.0008
Error	15	0.0525

*Significant at the 1% level.

The slope of the lines was 1.59, which was effectively the same as the earlier estimate, and there was no significant curvature. The error variance was also smaller than in the first group. The value of λ for the assay was 0.143 with a standard error of 0.03. The estimate of the ratio of potency of the unknown to the standard was 92% with a standard error of 11%. The true value for the unknown was 86% of the standard.

The value of λ obtained in this assay, 0.143, is somewhat better than most of the values previously reported. Stein, Jinks, and Mirsky (11) obtained a mean λ of 0.33. The same authors report that a value of 0.25 was calculated for the data given by Jeffers, Livezey, and Austin (12). Paulisch (5) compared several response measures including those proposed by Jeffers *et al.* (12) and Dicker (4). The values of λ that Paulisch reported were 0.22 for the method of Jeffers *et al.* and 0.19 for Dicker's procedure. Dettelbach (6) obtained a λ of 0.23 but notes in a footnote that with more recent data he has achieved a value of 0.09.

Discussion

Three important features of Dicker's method (4) for the bio-assay of ADH are the intravenous route of administration, the suppression of endogenous ADH secretion by the use of ethanol anaesthesia, and the automatic maintenance of water load throughout the assay. The first of these three features is now recognized by all workers in this field to be essential for maximum sensitivity, and has been retained in the present procedure. Maintenance of a reasonably constant water load is also essential, but it would appear that strict constancy is not required. Thus, manual replacement of the water in 0.5-ml amounts at intervals of a few minutes results in a urine flow as constant as that effected by automatic injection of smaller amounts at shorter intervals. Though more laborious for the operator, the extremely simple apparatus described here is so inexpensive that it can be set up in any laboratory. Moreover, the absence of complicated electronic and mechanical parts removes the possibility of instrumental failures.

The major difference between this procedure and that of Dicker lies in the use of tranquillization with perphenazine rather than anaesthesia with alcohol. The disadvantages of alcohol sedation have been described in the introduction; the only advantage over other types of anaesthesia is its reported ability to suppress endogenous ADH secretion. In the present method, the use of a conscious animal prevents the risk of circulatory depression. The performance of the major operative procedures on the day before the assay, the tranquillization of the animals with perphenazine, and the avoidance of any handling of the animals during the assay, appear to be quite effective in preventing ADH release due to extraneous factors. Preliminary trials with adrenal demedullated rats indicated that these animals, even when fully conscious, maintained a very constant urine output after hydration, despite a prolonged delay in attaining maximal diuresis. This observation suggested the possibility that animals tranquillized by perphenazine might be useful for the assay without the prolonged recovery necessary after adrenal demedullation. Kovacs *et al.* (13) have shown that chlorpromazine in low dosage does not affect water diuresis, but potentiates the effect of exogenous ADH. We have found that perphenazine, in the dosage used here, does not affect water diuresis; if it also potentiates the effect of ADH it should contribute to the sensitivity of this assay.

The statistical analysis of results obtained by this method indicates a high degree of sensitivity. The minimum dose of Pitressin capable of producing a

measurable response is approximately 25 μunits in a 200- to 250-g rat. This physiological sensitivity is of the same order as that claimed by Dicker (4), though not as great as that reported by Dettelbach (6). However, the value of this assay technique, in terms of its ability to determine accurately the relative potencies of standard and unknown (illustrated by the value of $\lambda = 0.143$), is greater than that of any other method published to date, with the exception of Dettelbach's (ref. 6, footnote p. 383). In view of the much greater complexity of his procedure, the present method has obvious advantages. Although a 3×2 assay design was used here, a 2×2 design would be adequate for routine use.

Various authors (14, 15) have called attention to the lack of specificity of change in urine volume as an index of ADH activity, and the advantage of measurement of urine density or solute content in this respect. Some doubt may arise concerning the validity of this view in relation to the assay of biological fluids or crude extracts containing substances other than ADH, which might produce non-specific effects upon urine density (16, 17). However, we have not investigated this matter experimentally, and cannot comment upon it at the present time. Certainly volume measurements as made in the present procedure are much simpler, with no evident loss of sensitivity. With the choice of urine volume change for assessing ADH effect, there remains the problem of choosing the best manner of expressing the effect. There are virtually as many indices as there are workers in this field. Most expressions of effect involve a comparison of pre- and post-injection flow rates, in terms of either the volume secreted in a standard time, or the time taken to secrete a standard volume. One publication (18) proposes the use of only the postinjection flow rate unrelated to the preinjection rate. However, until the mechanism of action of ADH is definitely known, no one index of antidiuretic effect is theoretically more justifiable than any other, and the choice must be an empirical one.

As mentioned previously, the statistical precision obtained by Dettelbach (6) was of the same order as that given by the present method. However, Dettelbach's calculation is based upon the urine flow in the 5-minute period beginning 1 minute after the injection of ADH. Our results indicate an appreciable variation between the patterns of response to different dose levels in a given rat. Thus, inhibition may not become apparent until 2 minutes after a 35-μunit dose yet be complete within 10 minutes, while it may begin at once after a 130-μunit dose yet last for up to 30 minutes. Therefore, we believe that it is more reasonable to use a calculation based on the entire period of inhibition.

Though the work reported here deals only with standard solutions of Pitressin, this method has already been applied successfully to the measurement of antidiuretic activity in urine extracts.

Acknowledgments

Technical assistance in the performance of the assays was provided by Mr. T. Bullivant, Mr. J. C. Kennedy, and Mr. P. Walpole.

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BIOCHEMICAL STUDIES ON SOCKEYE SALMON DURING SPAWNING MIGRATION

VII. STEROID HORMONES IN PLASMA[1]

D. R. IDLER, A. P. RONALD, AND P. J. SCHMIDT

Abstract

Cortisone, cortisol, and 17-hydroxyprogesterone were isolated from the plasma of prespawning Fraser River sockeye salmon. The female plasma contained double the concentration of cortisone and cortisol found in male plasma, 41 μg and 26 μg/100 ml respectively as compared with 22 μg and 11 μg/100 ml.

Corticosterone is probably present at a very low concentration and neither 11-desoxycorticosterone nor aldosterone was detected in the plasma from approximately 100 fish. Cortisone, cortisol, and total 17-hydroxysteroids were quantitatively determined in plasma samples obtained from two pure races of sockeye captured at various stages of sexual maturity. The combined concentration of cortisone and cortisol which, at the mouth of the river was three times that of the normal human, increased to as high as 17 times this level following spawning. The results are discussed in relation to the death of the fish.

Introduction

Cortisol has been conclusively established to be the principal adrenal cortical hormone in the peripheral plasma of the normal human (1). In general, total 17-hydroxysteroid determinations on human plasma have given higher values than have paper chromatographic methods involving the isolation of cortisol. A recent report describes a mean cortisol concentration in normal male plasma of 7.7 ± 3.5 μg/100 ml when cortisol was determined by paper chromatography as compared to 20.0 ± 8.7 μg/100 ml when total 17-hydroxysteroids were determined (2). Cortisol is also reported to be the principal steroid in the blood of several animals (3) and has been tentatively identified in carp plasma (4). Extracts of the plasma of the Atlantic salmon (*Salmo salar*) have been demonstrated to possess considerable glycogenic activity (5). The same investigators determined the total 17-hydroxycorticosteroid levels by the Nelson–Samuels methods and reported that there was considerably more glycogenic activity than could be accounted for on the basis of the chemical analytical procedure. Attempts to find cortisone in adrenal vein blood (6), where the level of cortisol is extremely high, and in human peripheral blood (7) have been unsuccessful. Occasional blood samples have been reported to contain small amounts of a "cortisone-like" substance but the evidence is inadequate to establish identity (8, 9).

The isolation of 17α-hydroxyprogesterone from the human ovary has been described (10).

The present report is concerned with the isolation and characterization of cortisol, cortisone, and 17-hydroxyprogesterone from sockeye salmon plasma

[1]Manuscript received April 10, 1959.

Contribution from the Fisheries Research Board of Canada, Chemistry Section of the Technological Station, Vancouver, British Columbia. A preliminary account of certain phases of this work has been published (Idler, D. R., Ronald, A. P., and Schmidt, P. J. J. Am. Chem. Soc. **81**, 1260 (1959)).

and with the quantitative determination of plasma steroids in sockeye at various stages of sexual maturity.

Experimental

Collection of Samples

All sockeye were captured with a dip net. The locations at which the various samples were taken are listed in Tables I and II. The isolation and characterization studies were carried out on the plasma obtained from several hundreds of female salmon captured October 28, 1958, near Lytton, British Columbia, where the fish were about one week from the spawning grounds.

The 1957 Stuart Lake fish were captured at Lillooet, 415 miles from the spawning area and on arrival on the spawning grounds at Forfar Creek, British Columbia (11). All fish were bled by severing the caudal artery and care was taken to exclude slime. Heparin was used as an anticoagulant for the samples taken on the Stuart Lake run while a mixture of ammonium and potassium oxalates was used on the Adams River samples. Samples were centrifuged immediately and the plasma was stored at the temperature of dry ice until analyzed. The temperature of the plasma samples did not exceed 4° C during the thawing process.

Purification of Materials

Solvents were reagent grade. All low-boiling solvents, chloroform, methanol, benzene, etc., were distilled through a suitable fractionating column to assure no residue on evaporation. Chloroform was always distilled immediately before use. Formamide and ethylene and propylene glycol were distilled under high vacuum. The distilled formamide was stored in a desiccator over concentrated sulphuric acid. Specially purified Whatman No. 1 filter paper was used for all paper chromatograms. The paper was extracted in a Soxhlet apparatus for 1 day with 5% ammonium hydroxide; this was followed by a 2-day extraction with 5% aqueous acetic acid, and a 3-day extraction with methanol. The paper was then dried *in vacuo* at a relatively low temperature and stored in Mylar plastic bags to exclude atmospheric contaminants.

Analytical Methods

The dihydroxyacetone side chain of the steroid hormones was determined with the phenylhydrazine reagent (12). The α-ketol determinations were carried out with blue tetrazolium as described by Mader and modified by Morris (8). Steroids with a Δ^4-3-ketone structure were detected on paper chromatograms with ultraviolet light and the location confirmed with the Δ^4-3-ketone specific alkali spray of Bush (1). The blue tetrazolium spray was used to detect steroids with the α-ketol side chain on paper chromatograms (13). The Zimmermann reagent was used to locate 17-keto steroids on paper chromatograms (14) and examination under ultraviolet light before and after alkali spray was used to locate those 17-keto steroids which also contained a Δ^4-3-ketone grouping. All spectra were recorded with a Beckman DK spectrophotometer. Semimicro (1 ml) cells were employed for most determinations.

Quantitative Analysis of Steroids

A fairly specific acid fluorescence method was used for the determination of cortisol and corticosterone (15). Quantitative determinations of cortisol and cortisone were also carried out with the Porter–Silber reagent. Total 17-hydroxysteroids were determined by the method of Silber and Porter. It was observed that a slight yellow color was formed immediately on the addition of the phenylhydrazine reagent to the plasma extracts. This color was not formed by the addition of acid alone nor was it produced by pure reference steroids, therefore a small additional correction not described in the published procedure was applied (12). The authentic steroids referred to are U.S.P. chromatographic reference standards.

Isolation and Characterization of Cortisone, Cortisol, and 17-Hydroxyprogesterone

In a typical isolation 1760 ml of plasma obtained from female sockeye captured at Lytton, B. C., was extracted four times with 2000-ml portions of ethyl acetate. The ethyl acetate was concentrated to 2000 ml in a flash evaporator at approximately 40° C with the receiver immersed in dry ice – acetone. The ethyl acetate (2000 ml) was washed three times with 100-ml portions of 5% aqueous sodium carbonate. The ethyl acetate was then washed with three 100-ml portions of water and the ethyl acetate phase clarified by the addition of small quantities of benzene (7). The residue was then taken to near dryness *in vacuo*. The residue was taken up in 300 ml of 70% aqueous methanol and extracted four times with equal volumes of *n*-hexane. The hexane phase was then washed with 200 ml of 70% methanol which in turn was washed four times with hexane. The methanol phases were combined and diluted with two volumes of water and the mixture was continuously extracted with peroxide-free ether for 24 hours.

Whatman No. 1 filter paper was impregnated with a 30% solution of formamide (16). The residue obtained after removal of the ether was taken up in a small volume of chloroform:methanol 1:1, divided in two portions, and each was applied at the origin on a 12-cm strip of paper. Cortisone, cortisol, and corticosterone were run as reference standards. The chromatograms were developed with chloroform:benzene 1:1 until the solvent had reached the bottom of the paper (17). A strip containing the standards and 1/40 of the plasma strips was examined under ultraviolet light, then sprayed with the alkali fluorescence reagent, and finally with the blue tetrazolium reagent. The plasma strips showed two strongly positive areas for all three tests. One corresponded with cortisol and the other with cortisone (or aldosterone). Visually cortisone appeared to be the major component. The remainder of the plasma paper strips were divided into six sections; one contained the origin and residual fatty material, two contained cortisol, three contained cortisone, four contained the region between cortisone and corticosterone, five corresponded to corticosterone, and the lower limit of corticosterone to the bottom of the paper was number 6.

The materials in sections 1 to 6 were eluted from the paper with three portions of hot methanol. Approximately 20-ml portions were used for the smaller

strips and 40 ml for the larger. The methanolic extracts were transferred to a titerlenmeyer (a 50-ml Erlenmeyer flask with a well 0.8 mm inside diameter and 3 cm deep attached to the bottom), and evaporated to dryness at 59° C under an atmosphere of nitrogen (18). The formamide was then removed under high vacuum at a temperature of about 70°. The contents of the flask were rinsed into the well with chloroform:methanol 1:1 and evaporated again under nitrogen. This procedure was repeated two or three times. The residue in the well was then taken up in several small portions of hot chloroform, the residual paper removed by filtration, and the filtrate was evaporated to dryness.

Fraction 2

Fraction 2 showed an absorption maximum at 239 mμ, and calculated on ϵ 15.8×10^3 and using the molecular weight of cortisol, there was 491 μg of steroid. However, by the more specific acid fluorescence method for cortisol there was only 311 μg or 63.4%. Fraction 2 was then run on paper in the toluene – propylene glycol system for 78 hours (13). Three ultraviolet-absorbing bands were then observed, 2A, 2B, and 2C. The major component was 2C and it had an R_f value identical with that of authentic cortisol. The solvents were removed as described above and substance 2C was shown to be cortisol as described below.

Fraction 3

Fraction 3 showed an absorption maximum at 237 mμ and, based on ϵ 15.8×10^3 and using the molecular weight of cortisone, there was 650 μg of steroid. Fraction 3 was rechromatographed in ethylene glycol – *n*-butylacetate along with authentic cortisone and aldosterone (16). A test strip showed that there was only one ultraviolet-absorbing compound and this corresponded in position to cortisone. This substance was also the only material which exhibited alkali fluorescence or reduced blue tetrazolium. The area corresponding to authentic aldosterone will be referred to as fraction 3A and the area corresponding to authentic cortisone as fraction 3B. Fraction 3A was rerun in toluene – propylene glycol and the aldosterone–cortisone area was bio-assayed for aldosterone activity as described below. Fraction 3B was homogeneous in the toluene – propylene glycol system and in 48 hours travelled exactly the same distance as cortisone.

Fraction 5

Corticosterone, if present, would be in fraction 5. The paper strip exhibited no visible ultraviolet absorption in this area, so to aid in the detection of corticosterone a further 400 ml of plasma was worked up as described above giving a total fraction 5 from 2160 ml of plasma. Fraction 5 was rechromatographed in toluene – propylene glycol and the area corresponding to authentic corticosterone (R_f 0.2) was cut out and eluted from the paper. The sample exhibited no maximum in the region of 240 mμ. The absorption due to the paper was linear and a calculation applying Allen's correction indicated a total of only 1.2 μg of ultraviolet-absorbing material as compared with an appropriate paper

blank (19). If this entire absorption was due to corticosterone it represented a level of only 0.05 μg/100 ml of plasma. The remainder of the paper contained no alkali fluorescent compounds.

Fraction 6

Even after hexane extraction the 70% methanol fractions always contained a certain amount of colored fatty material which ran near the solvent front in the chloroform–benzene system. Therefore fraction 6, which contained all steroids less polar than corticosterone was rerun in benzene: heptane 1:1 – formamide. Three ultraviolet-absorbing compounds were observed, only one of which will be considered in the present report. This material designated fraction 6B ran in a similar position to authentic 11-desoxycorticosterone (R_f 0.45). This material exhibited an absorption maximum at 239 mμ, and corresponded to 204 μg (19 μg/100 ml for 60% recovery) based on the constants for 11-desoxycorticosterone. However, the material was observed to exhibit a sulphuric acid chromogen different from 11-desoxycorticosterone (20). Fraction 6B was rechromatographed in normal hexane – propylene glycol and the ultraviolet-absorbing compound was found to have an R_f value relative to 11-desoxycorticosterone of 0.58, a value identical with that obtained for authentic 17α-hydroxyprogesterone. There was no ultraviolet-absorbing substance corresponding to the 11-desoxycorticosterone.

Identity of Fraction 2C with Cortisol and 3B with Cortisone

Once the chromatographic homogeneity of 2C and 3B had been established, a sample of each was treated for 2 hours at 22° C with concentrated H_2SO_4 (20). Substance 2C exhibited maxima at 237, 282, 395, and 478 mμ, and O.D. ratios of 0.98:1:0.47:0.55, and substance 3B exhibited maxima at 284, 343, and 420 and O.D. ratios of 1:0.38:0.31. These values were identical with those obtained from authentic cortisol and cortisone respectively. Aliquots of 2C and 3B gave quantitative Porter–Silber reactions for the dihydroxyacetone side chain of the magnitude expected from the concentration based on ultraviolet absorption measurements and the chromogens gave the expected wavelength maximum at 410 mμ. Authentic cortisol and 2C were treated with fuming sulphuric acid for 1.5 hours and the spectra of the resulting chromogens were identical, with absorption maxima at 240 mμ and 490 mμ and a shoulder at 270 mμ (21).

Oxidative Removal of the Ketol Side Chains of 2C and 3B

Sodium bismuthate (1 g) was added to ca. 30 μg of 2C, 3B, and authentic cortisol and cortisone, each of which was previously dissolved in 5 ml of 50% aqueous acetic acid. The mixtures were shaken for one-half hour and then filtered through purified Whatman No. 1 paper. The residues and papers were washed thoroughly with water. The aqueous acetic acid filtrates were extracted with chloroform. The chloroform was removed under a stream of nitrogen and the residues were run in heptane:benzene 1:1 – formamide. The reaction products of authentic cortisone and 3B produced a single ultraviolet-absorbing spot with the same R_f value as Δ^4-androstene-3,11,17-trione. Authentic cortisol and 2C both gave a major ultraviolet-absorbing substance correspond-

ing in R_f value to 11-β-hydroxy-Δ^4-androstene-3,17-dione, and both gave also a very minor component corresponding to Δ^4-androstene-3,11,17-trione. The major ultraviolet-absorbing components were eluted from the paper as described above and rerun in benzene:hexane:methanol:water 33:66:80:20 (1). The oxidation products of authentic cortisol and cortisone had R_f values of 0.16 and 0.23 respectively and both compounds absorbed in the ultraviolet and gave positive Zimmermann reactions. The oxidation products of substances 2C and 3B corresponded identically with those from the respective reference standards.

The infrared spectra of substances 2C and 3B confirmed their identity with cortisol and cortisone respectively.

Identity of Substance 6B with 17-Hydroxyprogesterone

A solution of substance 6B in methanol contained 18.3 μg/ml of 17α-hydroxyprogesterone as indicated by the ultraviolet absorption at 240 mμ. The solution of substance 6B and an equivalent solution of authentic 17α-hydroxyprogesterone gave the same quantitative Porter–Silber reactions with the wavelength maximum in each case at 340 mμ. The sulphuric acid chromogens produced by aliquots of the two solutions were also identical and exhibited absorption maxima at 285 and 430 mμ as compared to 285, 370, and 446 mμ for authentic 11-desoxycorticosterone. Substance 6B and authentic 17α-hydroxyprogesterone were each treated with 20 mg of sodium borohydride in 0.5 ml of 80% aqueous *tert*-butanol for 18 hours (10). The mixtures were then shaken with 1 g of sodium bismuthate in 4 ml of 50% aqueous acetic acid for 1 hour. The steroids were extracted as described above and chromatographed in the benzene:hexane:methanol:water solvent system. Substance 6B and 17α-hydroxyprogesterone both gave Δ^4-androstene-3,17-dione as the major product as well as three minor Zimmermann-positive substances with R_f values of 0.03, 0.17, and 0.35.

Substance 3A

The strip corresponding to authentic aldosterone from 2160 ml of plasma was applied as a spot and run in the toluene – propylene glycol system for 48 hours. There was no evidence for an ultraviolet-absorbing substance on the paper. The area corresponding to authentic aldosterone was cut out and extracted as described above. There was a negligible residue when the solvent was removed and when the fraction was run against an appropriate blank, there was no absorption maximum near 240 mμ. However, there was a shoulder in this region but if the total absorption was due to aldosterone it would represent less than 2 μg. The sample was taken up in ethanol and divided among six 5-day adrenalectomized rats. Three additional groups of eight rats were treated with 1, 4, or 16 μg of desoxycorticosterone acetate. The index of activity was the Na/K ratio in the urine sample collected 0–5 hours after injection. The sample proved to be virtually inactive giving a response less than that obtained with 1 μg of 11-desoxycorticosterone (DOCA). A sample of free aldosterone was also included in the assay at a dose of 0.25 μg and gave the expected response, with a potency of approximately 50$\times$DOCA (22).

Quantitative Determination of Steroids in Adams River Sockeye Plasma

Ethyl acetate extracts were prepared from 100-ml samples of the plasmas listed in Table I. The only modification from the large-scale isolation was in

TABLE I

Cortisone and cortisol in Adams River sockeye plasma

Date	No. fish	Location	Sex	Cortisone,* μg/100 ml	Cortisol,* μg/100 ml	Cortisol,† μg/100 ml	Total 17-hydroxysteroids, μg/100 ml‡
Oct. 16/58	34	Lower Fraser	M	—	—	8	—
Oct. 18/58	32	Lower Fraser	F	15	19	28	50
Oct. 27/58	21	Lower Fraser	M	27	12	11	33
	57	Lower Fraser	F	19	21	26	112 (62)§
Oct. 29/58	80	Lytton, B. C.	M	22	11	11	30
	80	Lytton, B. C.	F	41	26	24	126
Nov. 2/58	55	Adams Lake	M	27	64	66	154 (109)§
	15	"Spawned"	F	44	129	141	231

*Ethyl acetate plasma extracts purified by chromatography in chloroform:benzene 1:1 – formamide and toluene – propylene glycol and the steroids measured by the Porter–Silber reaction.

†Chloroform plasma extracts purified by chromatography in toluene – propylene glycol and the steroid measured by acid fluorescence.

‡Steroids determined directly on chloroform extracts of plasma with the Porter–Silber reagent.

§Because the Porter–Silber chromogen values did not agree with the sum of the cortisone and cortisol when the steroid levels were high, these samples were also assayed by the Eik-Nes modification of the Porter–Silber procedure (40).

the extraction of the 70% methanol fraction after the hexane partition. Three volumes of water were added to the 70% methanol and the steroids were extracted three times with 200-ml portions of chloroform. Each residue was then placed on a 14-cm length of prewashed Whatman No. 1 paper impregnated with formamide, and benzene:chloroform 1:1 was used as the developer. Cortisol, cortisone, and 17α-hydroxyprogesterone were run on an adjacent length of paper. The material from the plasma zone corresponding in position to authentic cortisol and cortisone was eluted from the paper as described above and run for 48 hours in the toluene – propylene glycol system. The areas of the plasma chromatogram corresponding to cortisone and cortisol were extracted from the paper, and made up to a suitable volume for analysis. Cortisol was analyzed both by acid fluorescence and the Porter–Silber reaction and the average taken while cortisone was analyzed by the latter method. The results are shown in Table I. Recovery experiments were carried out in duplicate with authentic standards which were added to 100-ml samples of Lytton male plasma at levels of 15 and 30 μg per 100 ml. The recovery of added cortisone through the entire procedure varied from 64 to 68% and averaged 65%. The recovery of added cortisol averaged 50%.

Quantitative Determination of Steroids in Prespawning Stuart Lake Sockeye Plasma

The plasma was pooled to give four samples from males and four from females (Table II). To 10 ml of each plasma 0.5 ml of 1 *N* sodium hydroxide was added and the mixture shaken and then extracted three times with 20-ml portions of chloroform (2). The chloroform extracts were chilled at 4° C overnight and filtered through glass wool. The chloroform was removed under nitrogen and the residue chromatographed in toluene – propylene glycol for 48 hours. The

TABLE II

Cortisol in Stuart Lake sockeye plasma

Location	Sex	No. fish	Cortisol, μg/100 ml	Sex	No. fish	Cortisol, μg/100 ml
Lillooet	M	10	18	F	10	42
Lillooet	M	10	27	F	10	37
Lillooet	M	10	18	F	10	37
Lillooet	M	10	21	F	—	—
			Av. 23			Av. 39
Forfar Creek	M	10	9	F	14	22
Forfar Creek	M	5	16	F	14	31
Forfar Creek	M	6	13	F	13	40
Forfar Creek	M	4	11	F	14	28
			Av. 13			Av. 30

area of the paper containing the cortisol was extracted with hot methanol and suitable aliquots were taken for acid fluorescence measurements.

The recovery of cortisol added to two of the samples treated as above was 65%. The results corrected for recovery are shown in Table II.

Discussion

The salmon pituitary has been shown to elaborate a substance with ACTH activity (23) and this gave rise to the hypothesis that the degenerative changes following spawning might be due to intolerable concentrations of adrenal cortical hormones produced under the influence of large amounts of ACTH (24). These studies have now demonstrated that Pacific salmon show extensive degeneration of the pituitary gland at full maturity and spawning, and this is accompanied by marked hyperplasia (and presumed increased secretory activity) of the adrenal gland. In contrast, mature rainbow trout, which spawn repeatedly, generally show little, if any, pituitary degeneration (25, 26, 27).

The greatly increased cortisol concentration in the plasma of both sexes following spawning is apparent by both chromatographic procedures, whether the final analysis was carried out by the Porter–Silber or the acid fluorescence method (Table I). The total 17-hydroxycorticosteroid levels as determined directly on chloroform extracts of plasma confirmed the greatly increased steroid content of the plasma following spawning. These data support the suggested high secretory activity of the adrenal gland at this stage in the life cycle. It appears plausible that these extremely high plasma hormone levels may play a role in the degenerative changes and death of the fish after spawning. Towards the completion of the present investigation a report appeared stating that several biochemical changes occurring in the plasma of an unidentified species of spawning Pacific salmon were consistent with adrenal hypercorticalism (28).

The presence of detectable quantities of cortisone was unexpected but even more surprising was the finding that it was a major plasma steroid in prespawning fish and predominated in several samples. Cortisol is the principal

plasma steroid in both sexes after completion of the spawning act. The data in Table I do not answer the one important question whether the increased cortisol level is coincident with the completion of the spawning act or whether there was a very significant increase between Lytton and spawning, a period of 13–15 days. Plasma samples were available from fish captured on arrival at the Stuart Lake spawning grounds (11). These fish were due to spawn in 4–5 days. Male plasma (100 ml) averaged 13 μg (Table II) as compared with 11 μg and female plasma averaged 30 μg as compared with 26 μg at Forfar Creek and Lytton respectively. These data suggest that there was a very sharp increase in the cortisol concentration in sockeye plasma during the brief period immediately preceding spawning and the completion of the spawning act. There appeared to be some decrease in plasma cortisol between Lillooet and Forfar Creek for the Stuart Lake sockeye. However, it should be emphasized that these samples were obtained in 1957 and, while they were stored at low temperature, some protein denaturation had occurred.

It is probably desirable at this time to interpret data on the Stuart Lake samples only to the point of indicating the order of magnitude of the cortisol concentration in plasma. The values may not be precisely comparable with those for the Adams River samples for the analyses of which the techniques were improved.

The total 17-hydroxysteroid levels are in most instances considerably higher than the combined cortisol and cortisone concentrations. There are many factors possibly contributing to this result, among which may be mentioned the contribution of 17-hydroxyprogesterone and the possible presence of non-steroid substances in the chloroform extracts of salmon plasma which react with phenylhydrazine. It has not yet been established whether the two steroids which accompany cortisol in the chloroform:benzene 1:1 – formamide system but separate from it in toluene – propylene glycol react with the Porter–Silber reagent. In any event, the total 17-hydroxycorticoid levels in human plasma have generally been found to be higher than the cortisol level even following chromatographic purification of the chloroform extracts (2).

The levels of cortisone and cortisol or the total 17-hydroxycorticosteroid levels found in the spawned fish or the total 17-hydroxycorticosteroid levels found in nearly all the female samples in the present study are sufficiently high to account for the glycogenic activity found in *Salmo salar* plasma (5). The report of this study was too abbreviated to permit an assessment of the possible reasons why the biological assay did not agree with the chemical (Porter–Silber) method. One conceivable explanation is that the less polar cortisone was not retained on the adsorbent along with the cortisol.

Another very important process which might be expected to be under hormone control during the spawning migration is the maintenance of electrolyte balance during and after the transition from salt to fresh water. Aldosterone is the most potent electrolyte-regulating hormone secreted by the human adrenal cortex. Aldosterone has been identified in the adrenal vein blood of hypophysectomized dogs (29). The level in normal human systemic blood is reported to be 0.08 μg/100 ml (30) and others report that sheep blood contains

0.35 μg/100 ml (31). Since aldosterone has a biological potency approximately 50 times that of 11-desoxycorticosterone in the assay method used in the present study, it would be expected that ca. 0.02–0.04 μg in the 2160-ml plasma sample could have been detected. If aldosterone were present at a concentration comparable with that in human plasma, approximately 1.7 μg should have been present, or 0.8–0.9 μg allowing for ca. 50% recovery.

Corticosterone has been reported to be the principal steroid in the circulating plasma of the rat (32) and is probably present at a level of approximately one-fifth that of cortisol in normal human plasma (8). However, only a very low concentration of corticosterone appears to be present in the female sockeye salmon plasma used in the present study. Both male and female sockeye salmon captured 415 miles from the spawning ground at Lillooet, B.C., on the 1956 Stuart Lake run and at Forfar, B. C. (Table II), were worked up as described in the experimental section and run in the toluene – propylene glycol system. The areas corresponding to authentic corticosterone were extracted from the paper and assayed by the fairly specific acid fluorescence method. The level of corticosterone was less than 1 μg/100 ml. Although it will be necessary to carry out studies on more samples before it can be concluded that the concentration of corticosterone is always extremely low in salmon plasma, it is highly unlikely that corticosterone plays a significant role in regulating ion balance. Another potent mineral-regulating hormone, 11-desoxycorticosterone, does not appear to be present in detectable quantities in human plasma or in the arterial blood of the dog. Small amounts (1.86 μg/100 ml) have been demonstrated in the adrenal blood of the dog (22). In the present study there was no evidence for the occurrence of 11-desoxycorticosterone in the large sample (1760 ml) of plasma. Therefore, none of the more probable electrolyte-regulating hormones was present in significant quantity in this salmon plasma.

Because of the relatively high levels of cortisol as compared with aldosterone in normal human plasma, it has been suggested that cortisol may play a significant role in maintaining normal mineral metabolism (30). If this conclusion is valid for human plasma, then it is indeed possible that the extremely high levels of cortisone and cortisol regulate mineral metabolism in salmon at least during the river migration.

The properties of substance 6B strongly suggest that it is identical with 17α-hydroxyprogesterone. The authors believe that this is the first reported isolation of this steroid from blood. The physiological effects of 17α-hydroxyprogesterone have only recently been investigated and a brief review may suggest some further line of research to the reader.

The efficacy of 17α-hydroxyprogesterone caproate and other progestogenic compounds has recently been discussed in detail (33). It is reported that 17α-hydroxyprogesterone has 60 times the progestational activity of progesterone in the Hooker-Forbes assay using Rockland-Swiss mice (34) but this compound gave negative results with the CHI strain (35). The progestational activity of 17α-hydroxyprogesterone in the Clauberg test was demonstrated to be increased greatly by esterification to the caproate (36). However, both 17α-hydroxyprogesterone and the caproate were significantly less active than

progesterone in the rat and mouse deciduoma and chick oviduct assays (37). The progestational activity of 17α-hydroxyprogesterone was reported to be much lower than that of progesterone when rabbits were the experimental animal. Neither 17α-hydroxyprogesterone nor the caproate exhibited significant glucocorticoid activity (38).

Progesterone has been measured in human placental blood and its metabolites Δ^4-pregnen-3-on,20β-ol and Δ^4-pregnen-3-on,20α-ol have been demonstrated in the human placenta (35). Progesterone-like activity has been reported in the plasma of oviparous snakes and it was suggested that its physiological importance was probably quite different from that of progesterone in mammals. The available evidence suggests that it is not concerned with gestation. However, there was an increase during gestation but the blood of the male with fully developed testes showed increased plasma levels (39).

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Addendum

As this paper was going to press, a report appeared, Hane, S. and Robertson, O. H. Proc. Natl. Acad. Sci. **45**, 886 (1959), on the Porter–Silber chromogen levels in king salmon plasma. These workers state that the ratio of cortisol to cortisone found by us for prespawning sockeye salmon was the reverse of that found for spawned king salmon. The present report shows that this is not a species difference but that the ratio of cortisol to cortisone found at Lytton does reverse following spawning. Hane and Robertson found no increase in the Porter–Silber chromogen material of plasma from spawned males. By contrast, the male sockeye examined in the present report showed both an increased Porter–Silber chromogen and cortisol concentration in the plasma.

INCREASED SENSITIVITY OF AN END-PLATE REGION TO ITS CHEMICAL MEDIATOR FOLLOWING DENERVATION OF ANOTHER END-PLATE REGION OF THE SAME CELL[1]

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With the technical assistance of BRUCE ERICKSON

Abstract

By using the sartorius muscle of the frog it has been shown that a normally innervated end plate develops an increased sensitivity to its normal transmitter following denervation of other end plates on the same cell. This increase in sensitivity was demonstrated by determining the concentration of acetylcholine which must be applied to a region of the muscle containing innervated end plates in order to produce a threshold contraction and also by recording the electrical and mechanical responses of the partially denervated muscle fibers to a single maximal stimulation of the muscle nerve. The results support the idea that the change which occurs in the denervated muscle fiber which renders it more sensitive to its normal transmitter is a change which involves the whole cell rather than the denervated end-plate region alone, and that some of this change is due to a decrease in cholinesterase activity of the muscle.

Introduction

There is little information concerning the sensitivity of the innervated end plate or subsynaptic membrane to its normal transmitter following denervation of other end plates or subsynaptic areas on the same cell. Increased responsiveness of nerve cells to presynaptic impulses, which develops several days after partial destruction of the presynaptic supply to these cells, is a common finding (1, 2, 3). One possible explanation of this phenomenon is an increased sensitivity of the postsynaptic cell to the transmitter released by the remaining presynaptic neurones. However, there is at present no direct evidence to support such an explanation.

The physiological change in the postsynaptic neurone or muscle cell which renders it more sensitive to various chemicals after complete removal of its nerve supply is still unknown. However, it is of considerable theoretical importance to know if this change is limited to the denervated end plate or subsynaptic region or if the whole cell is involved. For example, although the end-plate region of the frog sartorius muscle fiber is still at least 1000 times more sensitive to the action of applied acetylcholine than the rest of the membrane following denervation, there is some evidence that the sensitivity of the non-innervated portions of the membrane also increases (4, 5), which would indicate that the change which occurs following denervation is not limited to the denervated end-plate region of the muscle cell. This can be tested by determining the sensitivity of normally innervated end plates to their transmitter following denervation of other end plates on the same cell. A study of this type has been carried out and the results are presented below.

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Contribution from the Department of Pharmacology and Therapeutics, University of Manitoba Faculty of Medicine, Winnipeg, Manitoba.

Methods

Preparation

The sartorius muscle of the frog *Rana pipiens* was used in all experiments. The individual fibers extend the full length of this muscle and have two end-plate regions, one proximal and one distal, with a distinct nerve supply to each. Most, if not all, fibers have at least one neuromuscular junction in each end-plate region (6). The distribution of nerves on the inner surface of the muscle is illustrated diagrammatically in Fig. 1. The distally directed branch

FIG. 1. Diagrammatic representation of the distribution of nerve branches in the right sartorius muscle of the frog, as seen from the ventral surface.

closer to the edge at which the nerve enters the muscle lies on the surface. The other distal branch is generally embedded in the muscle. The latter branch is absent in some muscles; in others, two or more such branches are present.

Denervation

The frogs were anaesthetized with 1–2 ml of an 80 mg/ml urethane solution. All dissection was done under a dissecting microscope through an incision on the ventral surface of the thigh. For complete denervation the whole nerve was severed at its site of entry into the muscle. For partial denervation, the edge of the muscle opposite the site of nerve entry was freed from the adjacent tissue. The freed edge was lifted and turned over to expose the nerve supply. All branches running towards the distal end of the muscle were then cut. This procedure generally resulted in damage to some muscle fibers both on the edge used to lift the muscle and in the area where branches embedded in the muscle were cut.

Recording

Both sartorii were removed from each frog, leaving the proximal ends attached to the pelvic girdle. The muscle pair thus isolated was mounted vertically (Fig. 2). The distal end of each muscle was attached by a nylon thread to a critically balanced isotonic lever. The bath was filled or drained from below. This arrangement permitted the test solution to cover the proximal end-plate region without touching the distal end-plate zone.

Solutions

The solutions used throughout contained (in mmoles/l.): NaCl 111.3, KCl 2.5, $CaCl_2$ 1.08, $NaHCO_3$ 2.4, NaH_2PO_4 0.087, glucose 11.1. Except where noted, all solutions contained 10^{-6} g/ml of neostigmine bromide. Test solutions were prepared by adding appropriate amounts of acetylcholine bromide.

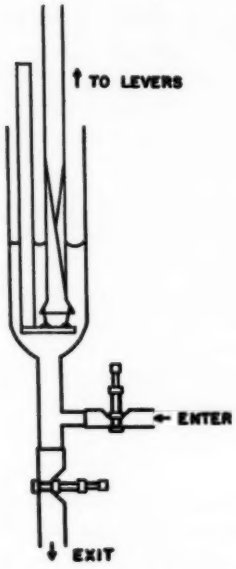

FIG. 2. Arrangement used for recording simultaneously the responses of both sartorii from a single frog to applied acetylcholine. For further details see text.

Procedure

The sensitivity of the muscles was tested by the application of acetylcholine solutions. The acetylcholine bromide concentration in the test solutions was increased in steps of approximately threefold (e.g., 10^{-7}, 3×10^{-7}, 10^{-6}, . . . g/ml). In a few of the earlier experiments steps of two- and five-fold (e.g., 10^{-7}, 5×10^{-7}, 10^{-6}, . . . g/ml) were used. Findings from experiments of the latter type have been included in the Results because they provide additional data without noticeably altering the results. The muscles were exposed to each test solution for at least 30 seconds. Between each application of the test solution the muscles were kept in an acetylcholine-free solution for at least 15 minutes when neostigmine was present and for at least 10 minutes when tests were made in the absence of neostigmine. Except when it was intended to test the sensitivity of the distal end-plate region, care was taken to be sure that the acetylcholine solution covered only the proximal end-plate region. This was done by keeping the upper level of the test solution in the bath below the point where the nerve entered the muscle. These tests were designed to find the minimum concentration of acetylcholine which would produce a discernible response of the muscle. This was considered the threshold concentration for the muscle provided the effect was reproducible and the muscle responded to the next higher concentration of acetylcholine.

In every frog in which a denervation was performed, the nerve supply to one of the sartorius muscles was left intact and this muscle served as a control. After every experiment with a partially denervated muscle, this muscle was

examined under a dissecting microscope to make sure that the nerve section had been properly performed, to exclude regeneration, and to ascertain that the proximally directed nerves were still viable. Functional integrity of the proximal nerves was determined by pinching the nerve with a pair of fine forceps, which resulted in a twitch of the whole muscle if the nerve was intact.

Statistical Analysis

The thresholds recorded were normally distributed only on a logarithmic scale. Statistical analysis by means of the Student *t* test was carried out using the logarithms of these values. On the other hand, the ratios of the threshold for the control to the threshold for the denervated muscle (threshold ratio) were distributed in a completely irregular manner. Statistical comparisons using these data were made by means of the "non-parametric" methods given by Snedecor (7). Because of the irregular distributions of the data, only the arithmetic means and the ranges have been given in Tables I and II.

Results

Sensitivity of the Sartorius Muscle to Applied Acetylcholine

The acetylcholine threshold in the presence of neostigmine was determined for muscle pairs from 22 unoperated frogs. Although the acetylcholine threshold differed for muscles from different frogs, in every case the paired muscles had the same threshold (Table I, "No denervation" group). Included in this group

TABLE I

Thresholds in the presence of neostigmine

	(*n*)		Control (C) ($\times 10^{-7}$ g ACh/ml)	Denervated (D) ($\times 10^{-7}$ g ACh/ml)	Ratio (C/D)
No denervation	24	Mean	35.3		1*
		Range	1–300		
Complete denervation	15	Mean	67.7	10.5	38.5
		Range	3–500	0.1–100	3–300
Partial denervation	17	Mean	28.0	6.5	13.9
		Range	3–100	0.03–30	1–100

*Right sartorius/left sartorius.

are the results obtained with muscles from two operated frogs in which it was discovered after testing that the nerves had not been cut at the time of operation.

Also shown in Table I are the results obtained with the unoperated control muscles from the two denervation groups. As expected, the mean thresholds for these two control groups do not differ significantly ($P > 0.2$) from that in the "No denervation" group of animals.

Effect of Complete Denervation on Sensitivity to Acetylcholine

The acetylcholine threshold in the presence of neostigmine was determined for 15 completely denervated muscles (Table I). These tests were performed 12 to 41 days following denervation. The threshold of each completely denerv-

ated muscle was lower than that of its mate from the same animal. As far as could be determined, the degree of sensitization was not related to the duration of the denervation within the period tested. It should be pointed out that the figures listed in the column headed "Ratio (C/D)" in Table I were obtained by adding the ratios for each muscle pair and dividing by the number of pairs in the group(n).

The degree of sensitization which we have found to follow complete denervation is less than is usually reported (4, 5). Two of our observations might help to explain this discrepancy. First, as is shown below, the presence of neostigmine tends to reduce the difference between the threshold of the denervated muscle and that of its mate. Second, the proximal end-plate regions of either partially or completely denervated muscles often appeared to respond to the application of acetylcholine concentrations well below their actual thresholds. These erratically occurring responses might well be confused with threshold responses unless further tests were performed. These aberrant responses were not observed in tests on unoperated control muscles.

Effect of Partial Denervation on Sensitivity to Acetylcholine

The sensitivity to applied acetylcholine of the proximal, innervated end-plate regions of 17 partially denervated muscles is shown in Table I. The tests were performed 12 to 58 days following denervation, and within this period the degree of sensitization was not related to the duration of denervation. The proximal, innervated end-plate zone of all but one of the 17 partially denervated muscles had a lower acetylcholine threshold than that of its mate. As can be seen from the threshold ratios (Table I), the completely denervated muscles as a group appeared to develop a greater sensitization than the partially denervated muscles. However, the thresholds were highly variable in both groups and this difference is not statistically significant ($P > .05$).

In four additional muscle pairs, which were tested more than two months after denervation, nerve regeneration had occurred. In one muscle pair tested 5 days after partial denervation, both muscles had the same acetylcholine threshold and the possibility had to be considered that sufficient time had not elapsed for the development of sensitization. Seven other muscle pairs were tested from 7 to 11 days following partial denervation and in all an increased sensitization of the denervated muscle was observed. The results obtained with these muscles are not included in the tabulated data.

Another interesting observation was that the distal, denervated end-plate zones of the partially denervated muscles did not have a lower threshold to applied acetylcholine than the proximal, innervated zones of the same muscles. This was demonstrated by determining the threshold with the entire muscle immersed in the test solutions. In only one of 24 muscles was a greater sensitivity detected and this was small (a threefold increase).

Influence of Neostigmine

Neostigmine was present in all solutions used in the tests reported above. This substance is known to be a potent inhibitor of cholinesterase and to have a direct depolarizing effect on the muscle end plate (8). Both of these effects are

likely to influence the experimental results, and consequently, tests were performed to obtain some estimate of their importance. In these experiments muscle pairs from 4 unoperated frogs, from 8 in which a muscle was partially denervated, and from 10 in which a muscle was completely denervated were used. After determining the threshold in neostigmine-free solutions, the muscle pairs were kept in a neostigmine solution for at least 30 minutes, and thresholds in the presence of neostigmine then determined.

The results are given in Table II. The presence of neostigmine reduced the acetylcholine threshold in all the muscles from unoperated frogs, a threefold

TABLE II

Effect of neostigmine treatment on threshold ratios

	(n)		Threshold ratio A Without neostigmine	Threshold ratio B With neostigmine	Ratio (A/B)
No denervation	4	Mean	1*	1*	1
		Range			
Complete denervation	10	Mean	243	42	9
		Range	10–1000	3–300	3–30
Partial denervation	8	Mean	37	18	3
		Range	3–100	1–100	1–10

*Right sartorius/left sartorius.

change in each muscle, but did not influence the threshold ratio. On the other hand, the use of neostigmine tended to reduce the ratio between the thresholds of the control and denervated muscle in each pair. In the case of the completely denervated muscles this reduction in the threshold ratios was large and statistically significant ($P < .05$), whereas in the case of the partially denervated muscles the reduction was small and not statistically significant ($P > .05$). It is also interesting to note that, as indicated by the threshold ratios, complete denervation resulted in a significantly greater sensitization ($P < .05$) than the partial denervation when these were tested in the absence of neostigmine. However, this difference in the sensitization produced by the two procedures disappeared when neostigmine was present in the solutions ($P > .05$).

Increased Response of the Partially Denervated Muscle to Nerve Stimulation

It was of interest to determine if the increased sensitivity of the innervated end plates to acetylcholine in the partially denervated muscle would result in an increased response of the muscle to nerve stimulation. Such an increased response was easily demonstrated. Thus the isometrically recorded twitch produced by a single maximal stimulation of the nerve was somewhat prolonged. However, this effect was more clearly seen in the electrical response of the postsynaptic cells, as is illustrated in Fig. 3. The electrical discharge was recorded by means of a steel-needle electrode tip (diameter $= 500\,\mu$) placed on the pelvic end of the muscle, a region free of end plates (4, 6). Since an electrode of this type records the activity of only a few muscle fibers in the

PLATE I

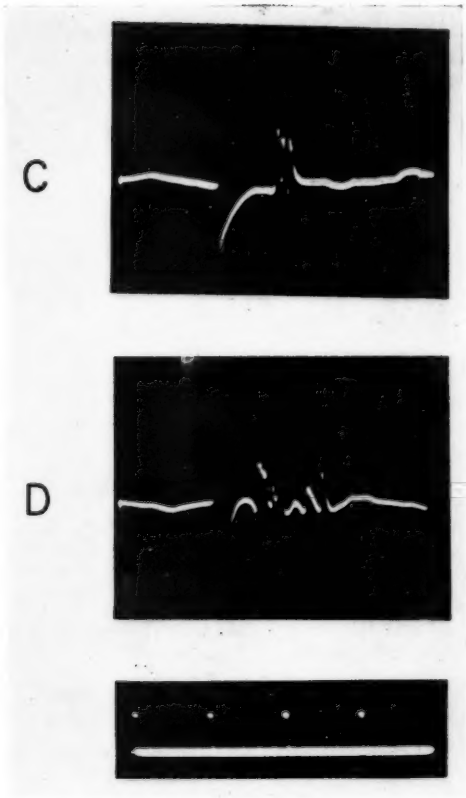

FIG. 3. Effect of partial denervation on the response of the sartorius muscle to a single maximal stimulation of its nerve. Action potentials of a few muscle fibers recorded by means of a steel-needle microelectrode placed on the pelvic, end-plate-free region of the muscles. C. Control muscle. D. Partially denervated muscle from the same frog 32 days after operation; time: 10 milliseconds between pulses.

immediate vicinity of the tip, the increased response seen in Fig. 3D was due to a repetitive response of some or all of these fibers to a single nerve impulse.

A lack of synchrony of this repetitive after-discharge in various muscle fibers might account for the fact that the twitch in the partially denervated muscle was only slightly larger than the twitch of the control muscle whereas the duration of the twitch of the denervated muscle was more than twice that of the control. These electrical and mechanical records were made with the muscle soaking in a solution having no neostigmine.

Discussion

The results presented above clearly show that normally innervated end plates of frog sartorius muscle fibers show an increased sensitivity to their normal transmitter following denervation of other end plates on the same cell. It has been stated that partial denervation does not have this effect (9), but no data were given to support this assertion. We are therefore unable to explain the discrepancy.

Partial denervation of the frog sartorius muscle results not only in an increased sensitivity of the normally innervated end plates to applied acetylcholine but also in an increased response to a single maximal nerve stimulation. These facts, along with the observation that the sensitivity of the denervated end plates is no greater than that of the innervated end plates on the same cells in the presence of neostigmine, make it hard to avoid the conclusion that the physiological change which occurs following denervation is a change involving the whole muscle cell.

We might expect that the presence of part of the nerve supply in the partially denervated muscle would tend to reduce the degree of sensitization. In the absence of neostigmine, a difference in the sensitization produced by partial and complete denervation was indeed observed, but this difference disappeared when neostigmine was used. This suggests that the sensitization which occurs following denervation can be ascribed to two processes. One is an increased responsiveness of the denervated muscle cell, which is apparently the same when either all or only some of the end-plate regions on the muscle cell are denervated. The other is a decrease in the cholinesterase activity of the muscle, a decrease which is more marked when all the end plates have been denervated.

The cause of the increased responsiveness of central nervous system neurones and of sympathetic ganglia to presynaptic impulses following partial destruction of their presynaptic nerve supply is still an open question. The results presented above showing that denervation of one or more end plates on a sartorius muscle fiber results in an increased sensitivity of other normally innervated end plates on the same cell to its chemical mediator may serve as a model for the processes which occur in the nervous system following partial removal of the presynaptic supply to a neurone and strongly support the idea that this increased responsiveness of central and autonomic neurones is a form of denervation sensitization. However, other mechanisms, such as induced branching of the remaining presynaptic fibers (2), have been proposed and may contribute to the increased responses.

Acknowledgment

I wish to thank Dr. Mark Nickerson and Dr. Ian Innes for their helpful criticisms and suggestions and Mr. Lowell Hurwitz for his help in carrying out some of the earlier experiments.

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